

(FILE 'HOME' ENTERED AT 16:11:46 ON 06 DEC 2005)

FILE 'MEDLINE' ENTERED AT 16:11:58 ON 06 DEC 2005

L1	2731 S THROMBOSPONDIN
L2	189 S L1 (20A) ANTIBOD?
L3	1 S L2 AND (NEURDEGEN? OR ALZHEIMER? OR DEMENTIA)
L4	0 S L2 AND BODY FLUID
L5	3 S L2 AND MAMMAL?
L6	113 S L2 AND BLOOD
L7	29 S L6 AND DETECT?

ANSWER 1 OF 1 MEDLINE on STN

AN 93035632 MEDLINE

DN PubMed ID: 1415477

TI Immunohistochemical identification of thrombospondin in normal human brain and in **Alzheimer's** disease.

AU Buee L; Hof P R; Roberts D D; Delacourte A; Morrison J H; Fillit H M

CS Department of Geriatrics and Adult Development, Mount Sinai School of Medicine, New York, New York.

NC AG 05138 (NIA)

AG 06647 (NIA)

SO American journal of pathology, (1992 Oct) 141 (4) 783-8.

Journal code: 0370502. ISSN: 0002-9440.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Abridged Index Medicus Journals; Priority Journals

EM 199211

ED Entered STN: 19930122

Last Updated on STN: 19980206

Entered Medline: 19921103

AB Thrombospondin is part of a family of adhesive glycoproteins and is involved in a number of physiologic processes such as angiogenesis and neurite outgrowth. Immunohistochemical localization of thrombospondin in normal human brains was investigated in the hippocampus and inferior temporal cortex. Two **antibodies** (one polyclonal and one monoclonal) against **thrombospondin**-labeled microvessels, glial cells, and a subpopulation of pyramidal neurons. The distribution of thrombospondin staining in patients with **Alzheimer's** disease was found to be comparable to control subjects. However, in patients with **Alzheimer's** disease a subset of pyramidal neurons that may be vulnerable in **Alzheimer's** disease exhibited decreased staining. This decrease in the intensity of labeling might constitute a marker for a neuronal population prone to early degeneration. In addition, thrombospondin staining was demonstrated in senile plaques in **Alzheimer's** disease. These results suggest that thrombospondin may be involved in the process of neuronal degeneration and senile plaque formation.

ANSWER 1 OF 3 MEDLINE on STN

AN 97094819 MEDLINE
 DN PubMed ID: 8940053
 TI Viral Myc oncoproteins in infected fibroblasts down-modulate thrombospondin-1, a possible tumor suppressor gene.
 AU Tikhonenko A T; Black D J; Linial M L
 CS Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104, USA.. atikhone@fred.fhcrc.org
 NC CA58809 (NCI)
 P30 CA15704 (NCI)
 SO Journal of biological chemistry, (1996 Nov 29) 271 (48) 30741-7.
 Journal code: 2985121R. ISSN: 0021-9258.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 OS GENBANK-U33174
 EM 199701
 ED Entered STN: 19970128
 Last Updated on STN: 19980206
 Entered Medline: 19970107
 AB We are interested in identifying the transcriptional targets of the Myc oncoproteins. To this end, we have fused Myc of the MC29 retrovirus with the rat glucocorticoid receptor. This chimeric protein requires dexamethasone to undergo nuclear translocation and achieve an active conformation. We employed a differential hybridization approach to identify mRNAs that are induced or repressed in infected avian fibroblasts in response to dexamethasone. This screen yielded one mRNA underrepresented in the dexamethasone-treated cells. In Myc-transformed cell clones, its level decreases 6-fold as early as 4 h and more than 30-fold after 32 h of exposure to the hormone. This mRNA was also down-regulated by recombinant Myc retroviruses in rodent fibroblasts, including those refractory to transformation. Sequence analysis revealed that it is homologous to the 3' untranslated regions of the **mammalian thrombospondin-1** genes. Using an anti-**thrombospondin antibody**, we confirmed that rodent cells overexpressing Myc produce very small amounts of this protein. Also, they do not support efficient expression of a reporter gene driven by the thrombospondin-1 promoter. Thus, thrombospondin-1 is a bona fide target of Myc. Moreover, its silencing might pertain to the transforming activity of Myc, since in several systems thrombospondin-1 exhibits tumor suppressor properties, presumably due to its negative effect on neovascularization.

L5 ANSWER 2 OF 3 MEDLINE on STN

AN 96149494 MEDLINE
 DN PubMed ID: 8557772
 TI Induction of transforming growth factor-beta autocrine activity by all-trans-retinoic acid and 1 alpha,25-dihydroxyvitamin D3 in NRP-152 rat prostatic epithelial cells.
 AU Danielpour D
 CS Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892, USA.
 SO Journal of cellular physiology, (1996 Jan) 166 (1) 231-9.
 Journal code: 0050222. ISSN: 0021-9541.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 EM 199602
 ED Entered STN: 19960312
 Last Updated on STN: 19980206
 Entered Medline: 19960226

AB Retinoids and vitamin D analogues are known to inhibit the proliferation of a variety of cells in culture and prevent the formation of certain tumors in **mammals**. Although it is well established that these hormones control the transcription of many genes upon binding to and activating specific nuclear receptors, the mechanisms by which they prevent cancer are as yet poorly understood. In this study the role of the transforming growth factor-beta (TGF-beta) growth inhibitors, in promoting the biological activities of all-trans-retinoic acid (RA) and 1 alpha,25-dihydroxyvitamin D3 (1,25-(OH)2D3) was studied in NRP-152 cells, a nontumorigenic epithelial line derived from rat dorsal-lateral prostate. Inhibition of growth by nanomolar concentrations of RA was associated with an increase in both mRNA and protein for all three TGF-beta isoforms, with greater and much earlier increases for TGF-beta s 2 and 3 (5.5 h) than for TGF-beta 1 (24 h). A monoclonal antibody against TGF-beta and TGF-beta 1 latency associated peptide (LAP), both of which neutralize all three TGF-beta isoforms, each block the ability of RA to inhibit growth of NRP-152 cells by > 95%. Neutralization of growth inhibition by isoform-specific antibodies suggested that all three TGF-beta s are involved in this effect. The ability of RA to upregulate fibronectin and **thrombospondin** expression in NRP-152 cells was also blocked by the monoclonal **antibody**. 1,25-(OH)2D3, which also induced TGF-beta s 2 and 3 but not TGF-beta 1, and their respective mRNAs, also induced fibronectin and thrombospondin through induction of TGF-beta. Thus, autocrine production of TGF-beta s may be a significant part of the mechanisms by which RA and 1,25-(OH)2D3 promote cellular differentiation.

ANSWER 1 OF 29 MEDLINE on STN
AN 2005471403 MEDLINE
DN PubMed ID: 15933059
TI Efficiency of curative and prophylactic treatment with rituximab in ADAMTS13-deficient thrombotic thrombocytopenic purpura: a study of 11 cases.
AU Fakhouri Fadi; Vernant Jean-Paul; Veyradier Agnes; Wolf Martine; Kaplanski Gilles; Binaut Raynald; Rieger Manfred; Scheifflinger Friedrich; Poullin Pascale; Deroure Benjamin; Delarue Richard; Lesavre Philippe; Vanhille Philippe; Hermine Olivier; Remuzzi Giuseppe; Grunfeld Jean-Pierre
CS Service de Nephrologie, Hopital Necker-Enfants Malades, 149 rue de Sevres, 75015 Paris, France.. fadi.fakhouri@nck.ap-hop-paris.fr
SO Blood, (2005 Sep 15) 106 (6) 1932-7. Electronic Publication: 2005-06-02. Journal code: 7603509. ISSN: 0006-4971.
CY United States
DT (CLINICAL TRIAL)
Journal; Article; (JOURNAL ARTICLE)
(MULTICENTER STUDY)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 200510
ED Entered STN: 20050907
Last Updated on STN: 20051012
Entered Medline: 20051011
AB Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disease that occurs mainly in young adults. Acquired cases are usually a result of **antibodies** directed against ADAMTS13 (a disintegrin-like and metalloprotease [reprolysin type] with **thrombospondin** type 1 motif 13), a protease that cleaves the von Willebrand factor multimers. Prognosis has been improved by plasma therapy, but some acute severe forms are refractory to this treatment and achieving a sustained remission is still a challenge in chronic relapsing forms. We therefore conducted a multicentric open-label prospective trial to test the efficacy of rituximab, an anti-B-cell monoclonal antibody, as a curative and prophylactic treatment in patients with TTP as a result of anti-ADAMTS13 antibodies. Six patients were included during an acute refractory TTP episode. Five patients with severe relapsing TTP and persistent anti-ADAMTS13 antibodies were prophylactically treated during remission. All patients received 4 weekly infusions of rituximab. The target of treatment was to restore a significant ADAMTS13 plasma activity (> 10%). Treatment with rituximab led to clinical remission in all cases of acute refractory TTP. In all patients, anti-ADAMTS13 antibodies disappeared, and a significant (18%-75%) plasma ADAMTS13 activity was **detected** following treatment. Tolerance of rituximab was good. Rituximab is a promising first-line immunosuppressive treatment in patients with acute refractory and severe relapsing TTP related to anti-ADAMTS13 antibodies.

L7 ANSWER 2 OF 29 MEDLINE on STN
AN 2004369952 MEDLINE
DN PubMed ID: 15272576
TI Biomarker in gynecologic malignancies.
AU Isonishi Seiji
CS Department of Obstetrics and Gynecology, The Jikei University School of Medicine, 3-25-8 Nishishinbashi, Minato-ku, Tokyo 105-8461, Japan.
SO Gan to kagaku ryoho. Cancer & chemotherapy, (2004 Jul) 31 (7) 1003-7. Ref: 13
Journal code: 7810034. ISSN: 0385-0684.
CY Japan
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LA Japanese
FS Priority Journals

EM 200407
ED Entered STN: 20040728
Last Updated on STN: 20040801
Entered Medline: 20040730
AB We selected ovarian cancer as being the most representative of the gynecologic malignancies since it has the largest number of tumor markers now in clinical use. First, variety of serum tumor markers were developed and regularly used to **detect** the existence of ovarian cancer and its stage. These markers of monoclonal antibodies could **detect** three different classes of cell surface antigen. CA 125, CA 130, CA 602 are antibodies raised against the core protein of the proteoglycan molecule, whereas CA 19-9, CA 50, KMO-1 and CA 72-4, STN, CA 546 are antibodies against a different portion of the glycosaminoglycan chain from the core protein. These markers, when combined with another group of tumor markers for discriminating the variety of pathological types of ovarian cancer, might have the potential to provide a better predictive value. Second, biomarkers for the **detection** of early-stage ovarian cancer are urgently needed and developed also in gynecologic tumors. These include a ovarian cancer-specific proteomic patterns generated by mass spectroscopy and single nucleotide polymorphism (SNP) digital analysis combined with assessment of allelic imbalance. Third, for monitoring the effect of treatment with cytostatic drugs, various types of biomarkers could be used as surrogate markers for the treatment depending on the mechanism of the effects of the drug used. For treatment with Bryostatin, a strong protein kinase C (PKC) stimulator, PKC activity promises to be an effective marker. For the trials with bevacizumab, anti-VEGF **antibody**, VEGF and its associated bFGF, CD-31, and **thrombospondin-1** (TSP-1), are leading candidates for monitoring markers.

L7 ANSWER 3 OF 29 MEDLINE on STN
AN 2004277835 MEDLINE
DN PubMed ID: 14976043
TI Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura.
AU Klaus Christoph; Plaimauer Barbara; Studt Jan-Dirk; Dorner Friedrich; Lammle Bernhard; Mannucci Pier Mannuccio; Scheiflinger Friedrich
CS Baxter BioScience, Biomedical Research Center, Uferstrasse 15, 2304 Orth/Donau, Austria.
SO Blood, (2004 Jun 15) 103 (12) 4514-9. Electronic Publication: 2004-02-19. Journal code: 7603509. ISSN: 0006-4971.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Abridged Index Medicus Journals; Priority Journals
EM 200407
ED Entered STN: 20040606
Last Updated on STN: 20040730
Entered Medline: 20040729
AB Severe deficiency of the von Willebrand factor (VWF)-cleaving protease ADAMTS13 can lead to thrombotic thrombocytopenic purpura (TTP), a disease associated with the widespread formation of platelet-rich thrombi in many organs. Autoantibodies that inactivate ADAMTS13 are the most frequent cause of acquired TTP. Little is known about epitope specificity and reactivity of anti-ADAMTS13 antibodies. In this study, a series of ADAMTS13 domains were expressed in Escherichia coli, and the reactivity of purified recombinant fragments with anti-ADAMTS13 auto-antibodies from 25 patients with severe ADAMTS13 deficiency was evaluated in vitro. All TTP plasmas contained antibodies directed against the cysteine-rich spacer (cys-rich/spacer) domain of ADAMTS13. In the plasma of 3 patients, antibodies were **detected** that reacted exclusively with the cys-rich/spacer domain, underscoring the importance of this region for functional activity of ADAMTS13. In 64% of the plasmas,

antibodies reacted with the 2 CUB domains, and in 56% they reacted with the isolated first **thrombospondin** type 1 (TSP-1) repeat and with the compound fragment consisting of the catalytic, the disintegrin-like, and the TSP1-1 domain. Less frequently, in 28% of the plasmas, antibodies reacted with the TSP1 repeats 2 to 8. Unexpectedly, antibodies reacted with the propeptide region in 20% of the plasmas. In conclusion, this study shows that even though anti-ADAMTS13 autoantibodies react with multiple domains of the protease, the cys-rich/spacer domain is consistently involved in antibody reactivity.

L7 ANSWER 4 OF 29 MEDLINE on STN
 AN 2004149643 MEDLINE
 DN PubMed ID: 15013968
 TI **Antibody** response of healthy adults to recombinant **thrombospondin**-related adhesive protein of cryptosporidium 1 after experimental exposure to cryptosporidium oocysts.
 AU Okhuysen Pablo C; Rogers G Aaron; Crisanti Andrea; Spano Furio; Huang David B; Chappell Cynthia L; Tzipori Saul
 CS Division of Infectious Diseases, The University of Texas Houston Medical School and School of Public Health, Houston, Texas 77030, USA..
 Pablo.C.Okhuysen@uth.tmc.edu
 NC FD-U-001621-01 (FDA)
 R01 AI 41735-01 (NIAID)
 RR-02558 (NCRR)
 SO Clinical and diagnostic laboratory immunology, (2004 Mar) 11 (2) 235-8.
 Journal code: 9421292. ISSN: 1071-412X.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 EM 200410
 ED Entered STN: 20040327
 Last Updated on STN: 20041019
 Entered Medline: 20041018
 AB Thrombospondin-related adhesive protein of Cryptosporidium 1 (TRAP-C1) belongs to a group of proteins that are also found in Toxoplasma gondii, Eimeria tenella, and Plasmodium species. TRAP-related proteins are needed for gliding motility, host-cell attachment, and invasion. The objective of this study was to characterize the antibody response to recombinant TRAP-C1 (rTRAP-C1) in healthy volunteers exposed to C. parvum and their association with clinical illness. A total of 31 healthy adult volunteers participated. Seven volunteers received the C. parvum TAMU isolate (inocula, 10 to 300 oocysts), and 24 volunteers received the C. parvum UCP isolate (500 to 10(5) oocysts). The total antibody (immunoglobulin M [IgM], IgG, and IgA) response to rTRAP C-1 was measured by enzyme-linked immunosorbent assays prior to and after exposure to Cryptosporidium parvum (days 0 to 45). Results of this study showed that individuals who were uninfected demonstrated higher reactivity at baseline compared to those who became infected. After challenge, increases in antibody reactivity were seen on days 30 and 45 compared to the results seen on days 0 to 5. The increases in antibody reactivity were statistically significant in subjects with diarrhea and with or without **detectable** oocysts compared to the results seen with those who were uninfected and asymptomatic. These findings suggest that increases in antibody reactivity to rTRAP-C1 occur after recent exposure to C. parvum.

L7 ANSWER 5 OF 29 MEDLINE on STN
 AN 2002205655 MEDLINE
 DN PubMed ID: 11939312
 TI Measurement of the activation of equine platelets by use of fluorescent-labeled annexin V, anti-human fibrinogen **antibody**, and anti-human **thrombospondin antibody**.
 AU Kingston Janene K; Bayly Warwick M; Sellon Debra C; Meyers Kenneth M;

Wardrop K Jane

CS Department of Veterinary Clinical Sciences, College of Veterinary
Medicine, Washington State University, Pullman 99164-6610, USA.

SO American journal of veterinary research, (2002 Apr) 63 (4) 513-9.
Journal code: 0375011. ISSN: 0002-9645.

CY United States

DT (EVALUATION STUDIES)

Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 200210

ED Entered STN: 20020410

Last Updated on STN: 20021022

Entered Medline: 20021021

AB OBJECTIVE: To investigate the potential use of fluorescent-labeled annexin V, anti-human fibrinogen **antibody**, and anti-human **thrombospondin antibody** for **detection** of the activation of equine platelets by use of flow cytometry. SAMPLE POPULATION: Platelets obtained from 6 Thoroughbreds. PROCEDURE: Flow cytometry was used to assess platelet activation as indicated by **detection** of binding of fluorescent-labeled annexin V, anti-human fibrinogen **antibody**, and anti-**thrombospondin antibody** to unactivated and ADP-, collagen-, platelet activating factor (PAF)-, and A23187-activated equine platelets. Human platelets were used as control samples. Determination of 14C-serotonin uptake and release was used to assess the extent of platelet secretion. RESULTS: Anti-human **thrombospondin antibody** failed to bind to equine platelets. Annexin V bound to platelets activated with PAF or A23187 when platelets had undergone secretion. Anti-human fibrinogen antibody bound to ADP-, PAF-, and A23817-activated platelets, but binding was not dependent on platelet secretion. The extent of binding of anti-fibrinogen antibody was less in equine platelets, compared with that for human platelets, despite maximal stimulation. CONCLUSIONS AND CLINICAL RELEVANCE: Activation of equine platelets can be **detected** by use of fluorescent-labeled annexin V and anti-human fibrinogen **antibody** but not by use of anti-human **thrombospondin antibody**. These flow cytometric techniques have the potential for **detection** of in vivo platelet activation in horses at risk of developing thrombotic disorders.

L7 ANSWER 6 OF 29 MEDLINE on STN

AN 2000460831 MEDLINE

DN PubMed ID: 10904102

TI A novel enzyme immunoassay for plasma thrombospondin. Comparison with beta-thromboglobulin as platelet activation marker in vitro and in vivo.

AU Bergseth G; Lappegard K T; Videm V; Mollnes T E

CS Department of Immunology and Transfusion Medicine, Trondheim, Norway.

SO Thrombosis research, (2000 Jul 1) 99 (1) 41-50.

Journal code: 0326377. ISSN: 0049-3848.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 200009

ED Entered STN: 20001005

Last Updated on STN: 20001005

Entered Medline: 20000922

AB A novel enzyme immunoassay for plasma **thrombospondin** (TSP) based on commercially available monoclonal **antibodies** was established. The following conditions for correct collection and preservation of **blood** samples were required: venipuncture directly into a vacutainer containing citrate, theophylline, adenosine and dipyridamole, storage on ice, and separation of plasma within 30 minutes. Thereafter,

the plasma TSP concentration remained constant at room temperature and after five times of freezing and thawing. Both inter- and intraassay variation coefficients were 5%. The lower **detection** limit was 20 microg/L. Median TSP concentration among 40 healthy **blood** donors was 43 microg/L, slightly lower than previously published. The assay is valid, reliable, and has certain advantages compared with previously published methods. TSP and beta-thromboglobulin (BTG) were then compared as platelet activation and biocompatibility markers in vivo: 23 patients undergoing cardiopulmonary bypass (CPB); and in vitro: effect of coating polyvinyl chloride with heparin. The kinetic patterns of TSP and BTG were markedly different in vivo but virtually identical in vitro, explained by different in vivo clearance mechanisms during CPB. We conclude that BTG is superior to TSP for evaluation of platelet activation during in vivo CPB, whereas TSP and BTG are virtually identical as markers in vitro.

L7 ANSWER 7 OF 29 MEDLINE on STN
 AN 2000295321 MEDLINE
 DN PubMed ID: 10828226
 TI Exposure of plasma proteins on Dacron and ePTFE vascular graft material in a perfusion model.
 AU Falkenback D; Lundberg F; Ribbe E; Ljungh A
 CS Department of Infectious Diseases and Medical Microbiology, Lund University, Lund, Sweden.
 SO European journal of vascular and endovascular surgery : official journal of the European Society for Vascular Surgery, (2000 May) 19 (5) 468-75. Journal code: 9512728. ISSN: 1078-5884.
 CY ENGLAND: United Kingdom
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 EM 200007
 ED Entered STN: 20000720
 Last Updated on STN: 20000720
 Entered Medline: 20000710
 AB OBJECTIVES: to compare the exposure of plasma proteins adsorbed onto three vascular graft materials (polytetrafluoroethylene ePTFE and two modifications of polyethyleneterephthalate Dacron). METHODS: surface exposure of fibronectin, vitronectin, **thrombospondin**, antithrombin III, IgG, high molecular-weight kininogen, fibrinogen, albumin and plasminogen was studied by incubation with radiolabelled **antibodies** in a perfusion model. Perfusion times with human plasma were 1, 4, 24 and 48 hours. RESULTS: all proteins could be **detected** at 1, 4, 24 and 48 hours after the start of perfusion. Overall, the least amount of proteins adsorbed onto ePTFE. CONCLUSIONS: the low adsorption of proteins onto ePTFE may be one of the reasons for the lower incidence of infections reported with this material. Copyright 2000 Harcourt Publishers Ltd.

L7 ANSWER 8 OF 29 MEDLINE on STN
 AN 1999223162 MEDLINE
 DN PubMed ID: 10208463
 TI Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate carcinoma.
 AU Giri D; Ittmann M
 CS Department of Pathology, Baylor College of Medicine, and Houston Department of Veterans Affairs Medical Center, TX 77030, USA.
 SO Human pathology, (1999 Apr) 30 (4) 419-24. Journal code: 9421547. ISSN: 0046-8177.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals

EM 199904
ED Entered STN: 19990511
Last Updated on STN: 19990511
Entered Medline: 19990427

AB The PTEN tumor suppressor gene encodes a dual-specificity protein phosphatase that may play a key role in modulating integrin-mediated signals. Inactivation of the PTEN gene has been **detected** in a small percentage of clinically localized prostate cancers but is common in metastatic disease. It has been shown in glioblastoma cell lines that loss of chromosome 10q, where the PTEN gene is located, is associated with increased angiogenic activity in the conditioned medium attributable to downregulation of thrombospondin-1, a negative regulator of angiogenesis. Therefore, we wished to determine whether inactivation of PTEN might be associated with increased angiogenesis in prostate cancers, because increased angiogenesis in localized cancers is associated with development of metastatic disease. Angiogenesis was assessed by counting microvessels in areas of maximal neovascularization after immunostaining with anti-factor VIII-related antigen antibodies in eight cases with proven homozygous deletion of the PTEN gene and 24 control cases. There was a statistically significant correlation between PTEN inactivation and increased microvessel counts. The microvessel density was higher at all Gleason scores in the cases with PTEN inactivation compared with control cases with the same score. To determine whether the increased angiogenesis in cases with PTEN inactivation was caused by downregulation of expression of the angiogenesis inhibitor **thrombospondin-1**, we analyzed a subset of the cases by immunostaining with anti-**thrombospondin-1 antibody**. Approximately 25% of cases showed decreased staining of prostate cancer cells, but there was no correlation with PTEN inactivation. Thus, PTEN inactivation is associated with increased angiogenesis, but the increased angiogenesis is not attributable to downregulation of thrombospondin-1 expression.

L7 ANSWER 9 OF 29 MEDLINE on STN
AN 1999214756 MEDLINE
DN PubMed ID: 10198567
TI Platelet-erythrocyte adhesion in sickle cell disease.
AU Wun T; Paglieroni T; Field C L; Welborn J; Cheung A; Walker N J; Tablin F
CS Division of Hematology-Oncology, UC Davis, School of Medicine, Sacramento 95817, USA.
SO Journal of investigative medicine : official publication of the American Federation for Clinical Research, (1999 Mar) 47 (3) 121-7.
Journal code: 9501229. ISSN: 1081-5589.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199905
ED Entered STN: 19990517
Last Updated on STN: 19990517
Entered Medline: 19990503

AB BACKGROUND: The abnormal adherence of sickle red **blood** cells (sRBC) to other cell types likely contributes to vaso-occlusion. Increased numbers of platelet-erythrocyte aggregates (PEA) and platelet activation have been described in sickle cell disease. The present study was undertaken to determine the contribution, if any, of the extracellular matrix protein thrombospondin to the adhesion of sRBC and platelets. METHODS: Platelet activation and PEA were measured using fluorescent-labeled monoclonal antibodies and flow cytometry. Platelet red-cell adhesion was measured by a gravity sedimentation assay. Erythrocyte-bound thrombospondin (TSP) was determined by enzyme-linked immunoabsorbant assay (ELISA). RESULTS: Our studies demonstrate significant platelet activation and adhesion of sRBC to platelets in sickle cell disease. Thrombospondin was **detected** on sRBC.

There was variable inhibition of sRBC-platelet adhesion by **antibodies** to CD36 (**thrombospondin** receptor) and **antibodies** to **thrombospondin**. CONCLUSIONS: Thrombospondin on sRBC may mediate, at least in part, sRBC-platelet adhesion in sickle cell disease. The study of heterotypic cell-cell interactions is important in understanding the pathogenesis of vaso-occlusion in sickle cell disease.

L7 ANSWER 10 OF 29 MEDLINE on STN
AN 1999201452 MEDLINE
DN PubMed ID: 10099113
TI Endogenous mucosal antiviral factors of the oral cavity.
AU Shugars D C
CS Departments of Dental Ecology and of Microbiology and Immunology, Schools of Dentistry and Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7455, USA.. diane_shugars@dentistry.unc.edu
NC DE-12162 (NIDCR)
DE-12166 (NIDCR)
SO Journal of infectious diseases, (1999 May) 179 Suppl 3 S431-5. Ref: 33
Journal code: 0413675. ISSN: 0022-1899.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LA English
FS Abridged Index Medicus Journals; Priority Journals; AIDS
EM 199906
ED Entered STN: 19990614
Last Updated on STN: 20000303
Entered Medline: 19990601
AB The oral cavity represents a unique site for mucosal transmission of human immunodeficiency virus type 1 (HIV-1). Unlike other mucosal sites, the oral cavity is rarely a site of HIV transmission despite **detectable** virus in saliva and oropharyngeal tissues of infected persons. One reason for this apparent paradox is the presence of endogenous mucosal antiviral factors. Innate inhibitory molecules, such as virus-specific **antibodies**, mucins, **thrombospondin**, and soluble proteins, have been identified and partially characterized from saliva. A recent addition to the growing list is secretory leukocyte protease inhibitor (SLPI), an approximately 12-kDa non-glycosylated protein found in serous secretions. Physiologic concentrations of SLPI potentially protect adherent monocytes and activated peripheral **blood** mononuclear cells against HIV-1 infection. SLPI levels in saliva and semen but not breast milk approximate levels required for inhibition in vitro. Characterization of SLPI and other endogenous antiviral molecules may enhance our understanding of factors influencing mucosal HIV-1 transmission.

L7 ANSWER 11 OF 29 MEDLINE on STN
AN 1999200586 MEDLINE
DN PubMed ID: 10102463
TI Circulating platelets show increased activation in patients with acute cerebral ischemia.
AU Zeller J A; Tschoepe D; Kessler C
CS Department of Neurology, Christian-Albrechts-University, Kiel, Germany.. j.zeller@neurologie.uni-kiel.de
SO Thrombosis and haemostasis, (1999 Mar) 81 (3) 373-7.
Journal code: 7608063. ISSN: 0340-6245.
CY GERMANY: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199906

ED Entered STN: 19990714
 Last Updated on STN: 20000303
 Entered Medline: 19990629

AB Platelet activation plays a central role in acute arterial stenosis as has been shown in coronary heart disease. Likewise it can be assumed to be of importance in the evolution of acute cerebral ischemia (ACI), particularly in patients with large vessel disease. Flow cytometric **detection** of platelet adhesion molecules as a marker of platelet activation in a group of patients with ACI and different etiologies has not been evaluated. In 72 patients with ACI and 72 controls, the exposure of activation-dependent adhesion molecules was determined using flow cytometry after immunostaining with monoclonal **antibodies** against CD 62, CD 63 and **thrombospondin**. The extent of platelet activation differed as a function of the etiology of ACI: platelets from patients with atherosclerosis of brain-supplying arteries expressed significantly more activation markers than did controls, whereas patients with cardioembolic stroke did not. By analyzing platelet adhesion molecules it is possible to describe platelet activation profiles in patients with acute cerebral ischemia. This diagnostic procedure will be useful for monitoring individualized anti-platelet therapy and may enable distinguishing different subgroups of stroke patients.

L7 ANSWER 12 OF 29 MEDLINE on STN
 AN 97430899 MEDLINE
 DN PubMed ID: 9284998
 TI Identification of thrombospondin as a high molecular mass protein released from activated equine platelets.
 AU Lipscomb D L; Boudreaux M K; Paxton R; Spano J; Welles E G; Schumacher J
 CS Department of Pathobiology, College of Veterinary Medicine, Auburn University.
 SO American journal of veterinary research, (1997 Sep) 58 (9) 954-60.
 Journal code: 0375011. ISSN: 0002-9645.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 EM 199710
 ED Entered STN: 19971024
 Last Updated on STN: 19980206
 Entered Medline: 19971016

AB OBJECTIVE: To establish the existence of platelet-derived proteins in equine plasma, with the future goal of developing an assay for the **detection** of in vivo platelet activation. ANIMALS: 5 mature healthy horses. PROCEDURE: Platelet-rich plasma and platelet-poor plasma were prepared from anticoagulated **blood**. Platelets were separated from plasma proteins by gel filtration, then activated with 0.5 microM platelet-activating factor. Protease inhibitors were added, and the released platelet proteins were harvested. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on the released platelet proteins and platelet-poor plasma, and the resultant silver-stained bands were compared. Immunoblot analysis was performed on released platelet proteins, using an **antibody** to human **thrombospondin**; human platelet-derived proteins served as the positive control for the **antibody**. RESULTS: Released platelet proteins in the presence of beta-mercaptoethanol (reduced samples) contained several proteins that were not observed in plasma including (mean +/- SEM) 194 +/- 2, 159 +/- 2, 151 +/- 2, 104 +/- 2, and 95 +/- 1 kd. Immunoblots of released platelet proteins had a prominent 180 +/- 2-kd protein in reduced samples that was recognized by an **antibody** to human **thrombospondin**, and with prolonged color development, 2 additional less prominent proteins (166 +/- 1 and 155 +/- 1 kd) were observed. CONCLUSIONS: Several proteins are released from activated equine platelets that are not **detectable** in normal equine

plasma. Thrombospondin is one of the high molecular mass proteins released by activated equine platelets. CLINICAL RELEVANCE: An assay can be developed for **detection** of thrombospondin in equine plasma and may be useful for **detection** of in vivo platelet activation in horses.

L7 ANSWER 13 OF 29 MEDLINE on STN
AN 97247086 MEDLINE
DN PubMed ID: 9121117
TI Scatter factor stimulates tumor growth and tumor angiogenesis in human breast cancers in the mammary fat pads of nude mice.
AU Lamszus K; Jin L; Fuchs A; Shi E; Chowdhury S; Yao Y; Polverini P J; Laterra J; Goldberg I D; Rosen E M
CS Department of Radiation Oncology, Long Island Jewish Medical Center, New Hyde Park, New York 11040, USA.
NC R01CA-64416 (NCI)
R01CA-64869 (NCI)
R01NS-32148 (NINDS)
SO Laboratory investigation; a journal of technical methods and pathology, (1997 Mar) 76 (3) 339-53.
Journal code: 0376617. ISSN: 0023-6837.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199704
ED Entered STN: 19970506
Last Updated on STN: 20000303
Entered Medline: 19970424
AB Scatter factor (SF) (also known as hepatocyte growth factor) is a plasminogen-related growth factor that induces tumor cell motility, invasion, and angiogenesis. Its receptor is a tyrosine kinase encoded by c-met, a protooncogene. Human breast cancer cells express SF and c-met in vivo; but human breast cancer cell lines do not produce SF in vitro. To determine whether SF can modulate the in vivo growth of human breast cancers within a natural mammary environment, we studied the orthotopic growth of SF-transfected (SF+) versus control (SF-) clones of MDAMB231 human mammary carcinoma cells in the mammary fat pads of athymic nude mice. SF+ clones expressed SF mRNA and produced very high titers of SF protein, whereas SF- clones did not express SF mRNA or produce **detectable** SF protein. Two SF+ clones (21 and 29) showed significantly increased tumor growth rates, reaching 3- to 4-fold larger primary tumor volumes and weights by time of killing ($p < 0.001$), as well as higher rates of axillary lymph node metastasis ($p < 0.02$), as compared with two SF- clones (32 and 34). In contrast, in vitro proliferation rates, two-dimensional colony formation, and soft agar colony formation were no greater in SF+ than in SF- clones. We performed further studies to investigate the discrepancy between the in vivo and in vitro growth results. Tumor extracts from SF+ clone (21 + 29) tumors had 50-fold higher SF content than did SF- clone (32 + 34) tumors, confirming high-level SF expression in vivo in SF+ tumors. Immunostaining of tumor sections for proliferating cell nuclear antigen revealed only a modest increase in the proportion of cycling cells in SF+ versus SF- tumors (70% versus 60%, respectively). The terminal deoxytransferase-labeling index was equally low (approximately 1%) in SF+ and SF- tumors, suggesting that apoptosis was not responsible for the slower growth of SF- tumors. However, SF+ tumors had significantly higher tumor microvessel densities than SF- tumors ($p < 0.001$). Moreover, there were much higher titers of chemotactic activity for microvascular endothelial cells in cell-conditioned media and primary tumor extracts from SF+ clones as compared with SF- clones. As demonstrated using the rat cornea assay, there was more angiogenic activity in SF+ tumor extracts than in SF- extracts. The increased chemotactic and angiogenic activities in SF+

tumor extracts were not explained by secondary alterations in the content of the angiogenic mediator, vascular endothelial growth factor, or the antiangiogenic glycoprotein, **thrombospondin-1**; and those activities were neutralized using an anti-SF monoclonal **antibody**. These findings suggest that SF promotes the orthotopic growth of human breast cancers, at least in part, by stimulating tumor angiogenesis.

L7 ANSWER 14 OF 29 MEDLINE on STN
 AN 97148721 MEDLINE
 DN PubMed ID: 9011589
 TI Adhesion and activation of platelets and polymorphonuclear granulocyte cells at TiO2 surfaces.
 AU Nygren H; Eriksson C; Lausmaa J
 CS Department of Anatomy and Cell Biology, University of Goteborg, Sweden.
 SO Journal of laboratory and clinical medicine, (1997 Jan) 129 (1) 35-46.
 Journal code: 0375375. ISSN: 0022-2143.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Abridged Index Medicus Journals; Priority Journals
 EM 199702
 ED Entered STN: 19970219
 Last Updated on STN: 19970219
 Entered Medline: 19970204
 AB The initial reactions of two TiO2 surfaces with **blood** were investigated by short-time exposure to capillary **blood** and analysis of surface-adsorbed plasma proteins and surface-adhering cells by using immunofluorescence techniques. Antibodies directed against platelet membrane antigen and P-selectin were used to visualize platelet adhesion and activation. Acridine orange and anti-CD11b were used to **detect** adhesion and activation of polymorphonuclear granulocytes (PMNs). **Antibodies** against **thrombospondin** were used as markers for platelet alpha-granules. The fluorescence intensity was quantitated by computer-aided image analysis. Commercially pure, polished sheet titanium was oxidized in two different ways: (1) the natural oxide was dissolved with hydrofluoric acid and a new oxide layer was grown by oxidation in nitric acid, or (2) annealing was performed at 700 degrees C in air. Auger electron spectroscopy and x-ray photoelectron spectroscopy showed that both surfaces had similar composition consisting of TiO2 covered by a carbonaceous surface contamination layer. The thickness of the oxide layer was 4 nm on the acid-oxidized surface and 39 nm on the annealed surface. Optical profilometry and scanning electron microscopy showed that the acid-oxidized surface was rough and the annealed surface was smooth. The fibrinogen/prothrombin-thrombin ratio in the initial protein film differed between the surfaces. The number of adhering platelets was larger at the surface with a high surface concentration of adsorbed fibrinogen. Platelet activation (CD62) and priming of PMNs (CD 11b) were also significantly higher on the acid-oxidized surface. The results indicate that non-self recognition of biomaterials is an array of transient reactions comprising protein-material, protein-cell, and cell-cell interactions.

L7 ANSWER 15 OF 29 MEDLINE on STN
 AN 96125021 MEDLINE
 DN PubMed ID: 8547640
 TI Human natural killer cell expansion is regulated by thrombospondin-mediated activation of transforming growth factor-beta 1 and independent accessory cell-derived contact and soluble factors.
 AU Pierson B A; Gupta K; Hu W S; Miller J S
 CS Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, USA.
 NC GM 08347-01A1 (NIGMS)
 P01-CA-21737 (NCI)

R01-CA-45814 (NCI)
 SO Blood, (1996 Jan 1) 87 (1) 180-9.
 Journal code: 7603509. ISSN: 0006-4971.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Abridged Index Medicus Journals; Priority Journals
 EM 199602
 ED Entered STN: 19960306
 Last Updated on STN: 19980206
 Entered Medline: 19960216
 AB Natural killer cells (NK) were studied to determine factors important in their expansion. Fluorescence-activated cell sorter (FACS) purified CD56+/CD3- NK cells cultured alone for 18 days in rIL-2 containing medium (1,000 U/mL) showed enhanced cytotoxicity but only minimal expansion. NK expansion was increased (12.5 +/- 1.6-fold) by coculturing NK with soluble factors produced by irradiated peripheral **blood** mononuclear cells (PBMNC) in which the two populations were separated by a microporous membrane. However, maximal NK expansion was always observed when NK were cocultured in direct contact with irradiated PBMNC (49.4 +/- 5.9-fold). To determine if marrow stroma, which supports differentiation of primitive NK progenitors, was a better accessory cell population than irradiated PBMNC, NK were cocultured in direct contact with primary marrow stromal layers. NK expansion with marrow stroma was similar to PBMNC. Fibroblast cell lines (M2-10B4, NRK-49F, NIH-3T3) and human umbilical vein endothelial cells (HUVEC), all homogeneous populations and devoid of monocytes, also exhibited a similar contact-dependent increase in NK expansion. Experiments were designed using fixed M2-10B4 stromal cells to separate the contact-induced proliferative stimuli from soluble factors. NK plated directly on ethanol/acetic acid-fixed M2-10B4, which leaves stromal ligands (cell membrane components and ECM) intact, resulted in increased NK expansion compared with medium alone. We further show that the combination of independent contact and soluble factors is responsible for maximal late NK expansion (days 28 through 40) but paradoxically inhibits early NK expansion (day 7). The proliferation inhibitory effects were verified by 3H-thymidine uptake and could be **detected** at days 2 through 6 but no longer 14 days after the initiation of the culture. We show that both laminin and thrombospondin inhibit early NK proliferation, whereas only thrombospondin was capable of also stimulating late NK expansion. The effect of thrombospondin on early NK proliferation is related to activation of transforming growth factor-beta 1 (TGF-beta) because anti-TGF-beta neutralizing **antibody** completely abrogated **thrombospondin**-mediated inhibition of early NK proliferation. Although inhibitory early in culture, active TGF-beta added only at culture initiation increases late NK expansion similar to thrombospondin. TGF-beta was not present in the thrombospondin preparation but latent TGF-beta in serum, or TGF-beta transcripts identified in IL-2-activated NK could explain paracrine or autocrine mechanisms for the regulation of NK proliferation. Finally, anti-TGF-beta neutralizing **antibody** only minimally affects stroma-mediated inhibition of early NK proliferation suggesting that aside from **thrombospondin** /TGF-beta, additional contact factors are important for the regulation of NK proliferation.

L7 ANSWER 16 OF 29 MEDLINE on STN
 AN 94283587 MEDLINE
 DN PubMed ID: 8013625
 TI Characterization of a novel monoclonal **antibody** (V58A4) raised against a recombinant NH2-terminal heparin-binding fragment of human endothelial cell **thrombospondin**.
 AU Morandi V; Edelman L; Legrand Y J; Legrand C
 CS INSERM U 353, Hopital Saint Louis, Paris, France.
 SO FEBS letters, (1994 Jun 13) 346 (2-3) 156-60.

Journal code: 0155157. ISSN: 0014-5793.

CY Netherlands

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 199407

ED Entered STN: 19940810

Last Updated on STN: 19980206

Entered Medline: 19940725

AB We report herein the characterization of a mouse monoclonal **antibody** (Mab) raised against the recombinant NH2-terminal heparin-binding domain (rHBD) of human endothelial cell **thrombospondin** (TSP). The antibody, a IgG1 (kappa), hereafter referred to as V58A4, reacted with two rHBD, TSPN18 and TSPN28 (i.e. 18 kDa and 28 kDa, respectively) with an affinity constant of 1.33×10^{-8} M. However, V58A4 failed to recognize native or deglycosylated forms of TSP purified from platelets or endothelial cells, as well as a 25-30 kDa HBD fragment produced by limited proteolysis of native TSP. In contrast, Mab V58A4 was shown to react with larger HBD fragments (50-60 kDa) that were present in platelet or endothelial cell extracts and could be retained on a heparin-Sepharose column at low salt concentrations. These fragments also reacted with MA-II, a mouse Mab (IgG1), which recognizes both rHBD and HBD as well as intact TSP. Thus, V58A4 Mab appears to selectively recognize naturally occurring HBD fragments of TSP and may thus prove to be useful for **detecting** TSP proteolysis in situ under various physiopathological conditions.

L7 ANSWER 17 OF 29 MEDLINE on STN

AN 94083630 MEDLINE

DN PubMed ID: 7505118

TI Integrin alpha 4 beta 1 and glycoprotein IV (CD36) are expressed on circulating reticulocytes in sickle cell anemia.

AU Joneckis C C; Ackley R L; Orringer E P; Wayner E A; Parise L V

CS Department of Pharmacology, University of North Carolina at Chapel Hill 27599-7365.

NC 1-RO1-HL45923 (NHLBI)

HL28391 (NHLBI)

RR00046 (NCRR)

+

SO Blood, (1993 Dec 15) 82 (12) 3548-55.

Journal code: 7603509. ISSN: 0006-4971.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Abridged Index Medicus Journals; Priority Journals

EM 199401

ED Entered STN: 19940209

Last Updated on STN: 19960129

Entered Medline: 19940124

AB The abnormal adherence of red **blood** cells, especially circulating reticulocytes (erythrocyte precursors), to the endothelium is believed to contribute to vascular occlusion observed in patients with sickle cell disease. Although several plasma proteins including von Willebrand factor and fibronectin have been proposed to mediate this adhesion, the mechanism of sickle cell adhesion to the endothelium remains unknown. Using flow cytometry, we screened sickle red **blood** cells with monoclonal antibodies (MoAbs) against known adhesion receptors and **detected** integrin subunits alpha 4 and beta 1 and the nonintegrin glycoprotein IV on reticulocytes but not on erythrocytes. No reactivity was **detected** against integrin subunits alpha 2, alpha 3, alpha 5, alpha 6, alpha v, beta 2, beta 3, integrin alpha IIb beta 3, or the nonintegrin glycoprotein Ib. Immunoprecipitation of reticulocytes with either alpha 4- or beta 1-specific antibodies identified the alpha 4

beta 1 complex (alpha 4(70) and alpha 4(80) forms), a receptor for fibronectin and vascular cell adhesion molecule-1. An **antibody** against glycoprotein IV, a receptor reported to bind **thrombospondin** and collagen, immunoprecipitated an 88-kD protein consistent with its reported M(r). MoAbs against alpha 4 and glycoprotein IV bound to an average of 4,600 and 17,500 sites per reticulocyte, respectively. Identification of alpha 4 beta 1 and glycoprotein IV on reticulocytes suggests both plasma-dependent and independent mechanisms of reticulocyte adhesion to endothelium and exposed extracellular matrix.

L7 ANSWER 18 OF 29 MEDLINE on STN

AN 94038669 MEDLINE

DN PubMed ID: 7693413

TI The in situ localization of tenascin splice variants and thrombospondin 2 mRNA in the avian embryo.

AU Tucker R P

CS Department of Neurobiology and Anatomy, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27157-1010.

SO Development (Cambridge, England), (1993 Jan) 117 (1) 347-58.
Journal code: 8701744. ISSN: 0950-1991.

CY ENGLAND: United Kingdom

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 199311

ED Entered STN: 19940117

Last Updated on STN: 19980206

Entered Medline: 19931129

AB Tenascin and thrombospondin belong to the growing family of extracellular matrix glycoproteins believed to have an anti-adhesive function during development. Immunohistochemistry has been used to identify these proteins in the developing central nervous system, in the matrix surrounding peripheral neurons, and in connective tissue. The **antibodies** used in most of these studies, however, could not distinguish between different splice variants (tenascin) nor different genetic forms (**thrombospondin**). For this reason, we used the reverse transcriptase polymerase chain reaction to generate DNA probes that are specific to the transcripts of high M(r) tenascin and thrombospondin 2. These probes were then used for an in situ hybridization study to determine the cellular origins of specific tenascin and thrombospondin forms throughout the development of the chick. The mRNA encoding high M(r) tenascin was found associated with motile cells and in tissues undergoing dynamic modeling: migrating glia, epithelial glia used as a substratum for migrating neurons, the growing tips of lung buds, and during osteogenesis. In contrast, the mRNAs of low M(r) tenascin were concentrated in areas of cartilage deposition and chondrocyte proliferation. Thrombospondin 2 mRNA was not **detected** in the developing central nervous system at any time during development by in situ hybridization. In contrast, it was found in embryonic mesenchyme, perichondrium, epimysium, and endothelial cells. Thrombospondin 2 mRNA was **detected** in poly(A) RNA isolated from embryonic spinal cord and cerebellum by polymerase chain reaction, though it was not **detected** in poly(A) RNA from the avascular retina. Thus, thrombospondin 2 mRNA may be present in the developing brain at low levels in endothelial cells or **blood** cells. These data support the notion that tenascin splice variants have distinct roles during development, and that thrombospondin 2 is more likely to be playing a role associated with the morphogenesis of connective tissue than neuronal development.

L7 ANSWER 19 OF 29 MEDLINE on STN

AN 94033514 MEDLINE

DN PubMed ID: 7693034

TI Ultrastructural demonstration of CD36 in the alpha-granule membrane of human platelets and megakaryocytes.
 AU Berger G; Caen J P; Berndt M C; Cramer E M
 CS INSERM U.348, Paris, France.
 SO Blood, (1993 Nov 15) 82 (10) 3034-44.
 Journal code: 7603509. ISSN: 0006-4971.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Abridged Index Medicus Journals; Priority Journals
 EM 199312
 ED Entered STN: 19940117
 Last Updated on STN: 19960129
 Entered Medline: 19931222
 AB CD36 (glycoprotein [GP] IV) is a membrane GP of 88 kD found on monocytes, endothelial cells, and platelets. It may serve as a receptor for collagen and is also able to bind **thrombospondin** (TSP), because a monoclonal **antibody** to CD36 inhibits TSP binding to thrombin-stimulated platelets. In the following study, we investigated the subcellular distribution of CD36 within normal resting platelets, thrombin-stimulated platelets, and in cultured megakaryocytes (MK) by an immunogold staining technique and electron microscopy. We used an affinity-purified monospecific polyclonal antibody showing a single major band of precipitation at 88 kD via immunoblot analysis. In normal platelets, ultrastructural observation **detected** immunolabeling for CD36, homogeneously distributed along the platelet plasma membrane and in the luminal side of the open canalicular system (OCS). Moreover, some labeling was found around the alpha-granules along the inner face of their limiting membrane. An average of 70% of granules were labeled. The granule-associated pool of CD36 was estimated at approximately 25% of the total cell content. To exclude the possibility of a cross-reaction with GPIIb-IIIa, platelets from a patient with type I Glanzmann's thrombasthenia (which completely lack GPIIb-IIIa) were studied and showed a similar subcellular distribution of CD36, including alpha-granule membrane labeling. In activated platelets, CD36 was shown to be redistributed to the OCS and pseudopods of the plasma membrane. Platelets from a patient with the Gray platelet syndrome expressed CD36 on their plasma membrane, and some immunolabeling was also found within small abnormal alpha-granules. In cultured MK, CD36 immunolabeling was **detected** in the Golgi saccules, associated vesicles, immature alpha-granules, and demarcation membranes. In conclusion, this study shows the existence of a significant intragranular pool of CD36 in platelets that may play a critical role in the surface expression of alpha-granule TSP during platelet activation.

L7 ANSWER 20 OF 29 MEDLINE on STN
 AN 93271114 MEDLINE
 DN PubMed ID: 8499406
 TI Platelet-associated factor XIII as a marker of platelet activation in patients with peripheral vascular disease.
 AU Devine D V; Andestad G; Nugent D; Carter C J
 CS Canadian Red Cross Blood Transfusion Service, Vancouver.
 SO Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association, (1993 Jun) 13 (6) 857-62.
 Journal code: 9101388. ISSN: 1049-8834.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 EM 199307
 ED Entered STN: 19930716
 Last Updated on STN: 19930716
 Entered Medline: 19930701

AB In the past several years, monoclonal antibodies have been developed that distinguish between resting and activated platelets in vitro. These **antibodies** recognize epitopes expressed on membrane proteins or soluble proteins, such as factor XIIIa and **thrombospondin**, that bind only to activated platelets. We have used fluorescence flow cytometry to determine whether three such antibodies can **detect** platelet activation in patients with severe peripheral vascular disease (PVD). Using two activation-specific monoclonal antibodies and a polyclonal antiserum to factor XIII a-chain, we have examined the platelets from PVD patients, age-matched control subjects who were free of **detectable** PVD, and unmatched control subjects. Cells analyzed as platelets were identified by their light-scatter profile and their reactivity with monoclonal anti-glycoprotein Ib. The platelets of patients with PVD showed no increase in binding of activation-dependent 1B3 (directed against a 180-kD membrane protein) compared with age-matched control subjects ($p = 0.780$). Similarly, there was no difference between PVD patients and control subjects in activation-dependent CD63 expression. Conversely, the binding of anti-factor XIII a-chain was significantly higher than in the control groups ($p < 0.001$). These data suggest that the **detection** of soluble factors that bind to activated but not resting platelets may be of use in the **detection** of pathological in vivo platelet activation.

L7 ANSWER 21 OF 29 MEDLINE on STN

AN 91230089 MEDLINE

DN PubMed ID: 2029507

TI Thrombin-induced platelet aggregates have a dynamic structure. Time-dependent redistribution of glycoprotein IIb-IIIa complexes and secreted adhesive proteins.

AU Heilmann E; Hourdille P; Pruvost A; Paponneau A; Nurden A T

CS URA 1464 CNRS, Pathologie Cellulaire de l'Hemostase, Hopital Cardiologique, Pessac, France.

SO Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association, (1991 May-Jun) 11 (3) 704-18.
Journal code: 9101388. ISSN: 1049-8834.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 199106

ED Entered STN: 19910707

Last Updated on STN: 19980206

Entered Medline: 19910619

AB The role of glycoprotein (GP) IIb-IIIa complexes and of adhesive proteins in mediating platelet aggregation is now well defined. However, less is known of the changes that occur once aggregation has begun. We report immunogold staining of thin sections of platelets or platelet aggregates, embedded in Lowicryl K4M, after the use of polyclonal **antibodies** to GP IIb or GP IIIa, fibrinogen (Fg), von Willebrand factor (vWF), and **thrombospondin** (TSP). Bound immunoglobulin G (IgG) was located by species-specific anti-IgG coupled to 5-nm gold particles and by electron microscopy. Initial experiments with platelet-rich plasma confirmed the feasibility of visualizing adhesive proteins between platelets in aggregates. Experiments then continued, using stirred suspensions of washed platelets incubated with alpha-thrombin. After 20 seconds, platelets were in contact without **detectable** release, although giant secretory vesicles containing adhesive proteins were seen. Internal pools of GP IIb-IIIa were progressively externalized within the aggregate. Secreted Fg was readily **detected** between platelets at 40 seconds. After 3 minutes, when most of the secretion had occurred, Fg had a patchwork-like distribution within the aggregate. After 6 minutes, zones with closely interspaced surface membranes, usually representing pseudopods, were dominant and Fg free. Results for vWF and TSP were

similar to those for Fg. Nonetheless, GP IIb-IIIa complexes continued to be located between adjacent surface membranes throughout the aggregate. Thrombin-induced platelet aggregates were isolated, and sodium dodecyl sulfate-soluble extracts were obtained. Western blot experiments showed that, although fibrinopeptide A had been cleaved, degradation of adhesive proteins by platelet proteases had not occurred. These results emphasize that a platelet aggregate is a dynamic structure and suggest that not all surface-contact interactions are mediated by Fg or the other adhesive proteins tested in this study.

L7 ANSWER 22 OF 29 MEDLINE on STN
AN 91228152 MEDLINE
DN PubMed ID: 1709309
TI Circulating activated platelets in myeloproliferative disorders.
AU Wehmeier A; Tschope D; Esser J; Menzel C; Nieuwenhuis H K; Schneider W
CS Department of Haematology, Oncology and Clinical Immunology,
Heinrich-Heine University, Dusseldorf, FRG.
SO Thrombosis research, (1991 Feb 1) 61 (3) 271-8.
Journal code: 0326377. ISSN: 0049-3848.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199106
ED Entered STN: 19910630
Last Updated on STN: 19980206
Entered Medline: 19910610
AB Platelet activation in patients with myeloproliferative disorders is often suggested by increased platelet alpha-granule secretion and an acquired storage pool defect of dense granules. To determine whether activated platelets circulate in patients with chronic myeloproliferative disorders, we evaluated the binding of monoclonal antibodies against activation-dependent epitopes on resting platelets (P 12, CD 63, and CD 62) in 12 patients with prominent megakaryocytic proliferation (8 patients with essential thrombocythemia, 2 with chronic myeloid leukemia, and 2 patients with polycythemia rubra vera). In addition, platelet aggregation in response to collagen, adenosine diphosphate, platelet activating factor, and agglutination with ristocetin was investigated. In 3 patients there was an increased percentage of platelets binding at least 1 activation marker. In 2 other patients, a trend towards increased antibody binding was observed. Binding of the **antibody** to **thrombospondin** (P 12) was related to expression of the GMP 140 protein (CD 62, $r = 0.76$, $p = 0.004$). There was no correlation of platelet aggregation defects in vitro to increased expression of platelet activation markers or to thrombohaemorrhagic complications. However, circulating activated platelets were **detected** in three out of five patients with a history of bleeding or thrombotic complications. The results of this preliminary study suggest that some but not all patients with myeloproliferative disorders showed increased amounts of circulating activated platelets. The relation of bleeding and thrombotic complications to the expression of activation-dependent epitopes on platelets in myeloproliferative disorders requires further investigation.

L7 ANSWER 23 OF 29 MEDLINE on STN
AN 90336469 MEDLINE
DN PubMed ID: 1696196
TI Flow-cytometric **detection** of surface membrane alterations and concomitant changes in the cytoskeletal actin status of activated platelets.
AU Tschoepe D; Spangenberg P; Esser J; Schwippert B; Kehrel B; Roesen P; Gries F A
CS Diabetes Research Institute, Heinrich-Heine University, Duesseldorf, Federal Republic of Germany.

SO Cytometry : journal of the Society for Analytical Cytology, (1990) 11 (5) 652-6.
 Journal code: 8102328. ISSN: 0196-4763.

CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 EM 199009
 ED Entered STN: 19901012
 Last Updated on STN: 19980206
 Entered Medline: 19900913

AB Occlusive vascular diseases are promoted by a "prethrombotic state" with increased platelet activity. Polymerization of cytoskeletal proteins and exposure of subcellular structures or rebinding of secreted proteins have been characterized as early reactions after platelet activation preceding adhesion and aggregation. Here, we demonstrate the kinetic increase in specific binding of monoclonal **antibodies** to **thrombospondin** (P10) and to platelet membrane activation markers CD63 (GP53, a 53 kD lysosomal protein) and CD62 (GMP140, a 140 kD alpha granule protein) by using a flow-cytometric bio-assay and the related change in the actin status by using the DNase-I inhibition assay after stimulation of normal human platelets with 0.2 U/ml thrombin. F-actin was raised from 41% to 51% of total platelet actin content 30 s after stimulation and remained thereafter constant (50% at 60 s). Simultaneously, the percentage of P10, CD63, and CD62 positive platelets was elevated from 5.4%, 24.4%, and 9.1% to 67.4%, 80.2%, and 82.3% respectively. The mean number of P10, CD63, and CD62 antibody binding sites increased from 3,300, 1,715, and 2,146 to 6,400, 6,800, and 9,016 per platelet. Conclusively, changes in the organization of the cytoskeletal protein "actin" and exposure of subcellular structures indicating platelet secretion can be regarded as markers of early platelet activation. Thus, the parallel response in both analytical systems provides further support for the diagnostic concept of flow-cytometric **detection** of preactivated platelets in the peripheral **blood** by using fluochrome staining procedures **detecting** activation dependent structural alterations directly at the cellular level.

L7 ANSWER 24 OF 29 MEDLINE on STN
 AN 90023587 MEDLINE
 DN PubMed ID: 2529765
 TI Immune-complex vasculitis: role of complement and IgG-Fc receptor functions.
 AU Smiley J D; Moore S E Jr
 CS Department of Medicine, University of Texas, Southwestern Medical Center, Dallas.
 SO American journal of the medical sciences, (1989 Oct) 298 (4) 267-77. Ref: 94
 Journal code: 0370506. ISSN: 0002-9629.

CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 General Review; (REVIEW)
 LA English
 FS Abridged Index Medicus Journals; Priority Journals
 EM 198911
 ED Entered STN: 19900328
 Last Updated on STN: 19900328
 Entered Medline: 19891117

AB Vasculitis contributes a major component to the pathogenesis of rheumatic diseases and glomerulonephritis. A common feature of these diseases is the presence of serum immune complexes (IC) which may be deposited in **blood** vessel walls. The modification of the size and solubility of IC by the classical and alternative complement pathways, and the recent

demonstration of the role of cellular complement receptors and IgG-Fc receptors in the handling of IC, now allow a better understanding of the pathogenesis of the severe forms of vasculitis. When complement deficiencies are present, the handling of IC is impaired, and vasculitis results. New **blood** tests for Factor VIII-related antigen, alkaline ribonuclease, plasma **thrombospondin**, and anti-neutrophil cytoplasmic **antibody** correlate with the presence of selected types of vasculitis. In addition, tissue thromboplastin release after application of defined tourniquet pressure can also **detect** subtle **blood** vessel injury. These new tests may allow diagnosis without risky organ biopsies. Advances in the diagnosis and treatment of vasculitis will also be discussed.

L7 ANSWER 25 OF 29 MEDLINE on STN
 AN 88111699 MEDLINE
 DN PubMed ID: 3123235
 TI Use of a monoclonal **antibody** to measure the surface expression of **thrombospondin** following platelet activation.
 AU Legrand C; Dubernard V; Kieffer N; Nurden A T
 CS U-150 INSERM/UA 334 CNRS, Hopital Lariboisiere, Paris, France.
 SO European journal of biochemistry / FEBS, (1988 Jan 15) 171 (1-2) 393-9.
 Journal code: 0107600. ISSN: 0014-2956.
 CY GERMANY, WEST: Germany, Federal Republic of
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 EM 198803
 ED Entered STN: 19900305
 Last Updated on STN: 19980206
 Entered Medline: 19880310
 AB The radiolabelled monoclonal **antibody**, 5G11, directed against native **thrombospondin**, has been used to assess the surface expression of secreted **thrombospondin** on human **blood** platelets. Emphasis has been placed on studying the role of fibrinogen in this process. Unstimulated platelets bound low amounts of 5G11 (about 2000 molecules/platelet). Binding increased 2-fold and 5-7-fold after stimulation of platelets with ADP or thrombin (or ionophore A23187) respectively. Unstimulated platelets from patients deficient in alpha-granule proteins (gray platelet syndrome) bound baseline levels of 5G11. However, binding was not increased after activation. Thrombospondin expression on thrombin-stimulated normal platelets was for a large part divalent-cation-dependent and was not affected by AP-2, a monoclonal antibody to GPIIb-IIIa complexes. However, binding of 5G11 was some 50% lower when platelets were stimulated in the presence of Fab fragments of a polyclonal rabbit antibody to fibrinogen. This suggested either a direct binding of thrombospondin to surface-bound fibrinogen or a steric inhibition due to a close proximity of the two proteins. The fact that binding of 5G11 was at the lower limit of the normal range to the stimulated platelets of an afibrinogenemic patient specifically lacking **detectable** fibrinogen favoured the latter explanation. Thus, a major fibrinogen-independent pathway for thrombospondin expression must exist.

L7 ANSWER 26 OF 29 MEDLINE on STN
 AN 87004677 MEDLINE
 DN PubMed ID: 3758079
 TI Structural and immunological comparison of human thrombospondins isolated from platelets and from culture supernatants of endothelial cells and fibroblasts. Evidence for a thrombospondin polymorphism.
 AU Clezardin P; Hunter N R; Lawler J W; Pratt D A; McGregor J L; Pepper D S; Dawes J
 SO European journal of biochemistry / FEBS, (1986 Sep 15) 159 (3) 569-79.
 Journal code: 0107600. ISSN: 0014-2956.

CY GERMANY, WEST: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198611
ED Entered STN: 19900302

Last Updated on STN: 20000303

Entered Medline: 19861107

AB Thrombospondin is a 450-kDa glycoprotein secreted by a variety of cells including endothelial cells, fibroblasts and platelets. The aim of this study was to compare the structural and immunological properties of human endothelial, fibroblast and platelet thrombospondins. All three thrombospondins were purified, digested with thermolysin, and the subsequent thermolysin-generated fragments isolated on a Superose 12 gel-permeation column using non-denaturing conditions. Each isolated proteolytic fragment of **thrombospondins** was then **detected** using either a radioimmunoassay with a polyclonal **antibody** or an enzyme-linked immunosorbent assay with three monoclonal **antibodies** (P10, MA-I, MA-II) directed against different epitopes of whole platelet **thrombospondin**. The fragmentation pattern of human endothelial thrombospondin consists of six major thermolysin-generated fragments (135-110, 98-82, 54-47, 25-20, 18-15 and 10 kDa) having molecular masses very similar to those observed with human fibroblast thrombospondin (115-100, 92-80, 54-49, 27-21, 17-13 and 12-10 kDa). Treatment of platelet thrombospondin with thermolysin only generated four proteolytic fragments having molecular masses of 110, 50, 25 and 12/10 kDa respectively. All these proteolytic fragments of endothelial, fibroblast and platelet **thrombospondins** were recognized by a polyclonal **antibody**. Monoclonal antibodies MA-I and P10 essentially recognized two proteolytic fragments (135-110, 98-82 kDa) of endothelial and fibroblast (115-100, 92-80 kDa) thrombospondins, and the 110-kDa fragment of platelet thrombospondin. Monoclonal antibody MA-II recognized three proteolytic fragments (54-47, 25-20, 18-15 kDa) of endothelial and fibroblast (54-49, 27-21, 17-13 kDa) thrombospondins, and two fragments (50, 25 kDa) of platelet thrombospondin, different from those **detected** by P10 and MA-I. The results clearly demonstrate that, under non-denaturing conditions, endothelial and fibroblast thrombospondins are structurally different from platelet thrombospondin since two fragments of endothelial thrombospondin (98-82, 18-15 kDa), equivalent to those of fibroblast thrombospondin (92-80, 17-13 kDa), are not released from platelet thrombospondin after thermolysin treatment. These three forms of thrombospondin are, however, immunologically indistinguishable. To investigate further the structural differences observed between platelet and the two other forms of thrombospondin, their degree of polymerization was compared. Prior to thermolysin treatment, the three forms of thrombospondin were separated into several oligomers ranging from 450 kDa to 3300 kDa when injected onto a Superose 6 gel-permeation column. (ABSTRACT TRUNCATED AT 400 WORDS)

L7 ANSWER 27 OF 29 MEDLINE on STN

AN 86104718 MEDLINE

DN PubMed ID: 3510684

TI Thrombospondin in essential thrombocythemia.

AU Lawler J; Cohen A M; Chao F C; Moriarty D J

NC HL 28749 (NHLBI)

SO Blood, (1986 Feb) 67 (2) 555-8.

Journal code: 7603509. ISSN: 0006-4971.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Abridged Index Medicus Journals; Priority Journals

EM 198603

ED Entered STN: 19900321

Last Updated on STN: 19980206

Entered Medline: 19860313

AB Essential thrombocythemia is a myeloproliferative disorder characterized by frequent bleeding and thrombotic complications. On a molecular level, two abnormalities of platelet thrombospondin have been identified: abnormal glycosylation of the intact 185,000-dalton chain has been **detected** and a shortened form of the thrombospondin chain is present. We have used two monoclonal **antibodies** and Lens culinaris lectin to probe the structure of **thrombospondin** in the platelets from three patients with essential thrombocythemia; one patient with polycythemia vera and two patients with secondary thrombocytosis. The presence of abnormal thrombospondin fragments with molecular weights of 160,000 and 30,000 was **detected** in the intact platelets and in the supernatant from thrombin-treated platelets, in all of the individuals except one of the secondary thrombocytosis patients. Monoclonal antibody binding studies indicate that both fragments are produced by proteolysis at a single site, which results in the removal of a 30,000-dalton fragment from the NH2-terminal. Lens culinaris lectin-binding studies revealed that some of the carbohydrate moieties of thrombospondin are near this cleavage site. The results are consistent with the hypothesis that the abnormal thrombospondin fragments observed under conditions of increased platelet production are due to increased susceptibility to proteolysis which, in turn, may be due to defective glycosylation.

L7 ANSWER 28 OF 29 MEDLINE on STN

AN 86026764 MEDLINE

DN PubMed ID: 3902120

TI Localization of thrombospondin in clots formed in situ.

AU Murphy-Ullrich J E; Mosher D F

NC HL 29586 (NHLBI)

SO Blood, (1985 Nov) 66 (5) 1098-104.

Journal code: 7603509. ISSN: 0006-4971.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Abridged Index Medicus Journals; Priority Journals

EM 198512

ED Entered STN: 19900321

Last Updated on STN: 19980206

Entered Medline: 19851206

AB Thrombospondin is a principal glycoprotein secreted by thrombin-stimulated platelets and has known affinities for fibrinogen and fibrin. We studied the distribution of thrombospondin in clots formed in situ on Formvar-coated coverslips at 37 degrees C for intervals up to 17 hours. The distributions of three other major platelet granular proteins--fibrinogen, fibronectin, and von Willebrand factor (vWF)--were also determined. The portions of the clots adhering to the coverslips after stripping, washing, and fixation with formaldehyde were stained for the four proteins by the peroxidase-antiperoxidase technique. Monoclonal **antibodies** were used to localize **thrombospondin**, fibronectin, and vWF; affinity-purified polyclonal **antibodies** were used to localize fibrinogen. Platelets stained positively for all four proteins. Thrombospondin was maximally present in the fibrin meshwork from 1 1/2 to 2 hours, after which the intensity of staining decreased until only trace amounts of thrombospondin were **detectable** between four and 17 hours. Antifibrinogen and, to a lesser extent, antifibronectin stained the fibrin meshwork at all time points. The vWF was not **detectable** in the fibrin meshwork at any time point. Staining of polymorphonuclear leukocytes (PMNLs) in a fine granular pattern was found with antithrombospondin. The fraction of PMNLs staining positively was 6% to 14% at 1/2 to 4 hours and increased at eight hours to 27%. At 17 hours, 52% of the PMNLs stained for

thrombospondin. More than 48% of the PMNLs stained with antifibrinogen at all time points. PMNLs did not stain for either fibronectin or vWF. These studies indicate that thrombospondin is a transient component of the temporary fibrin meshwork and has a unique spatial and temporal distribution in the hemostatic plug.

L7 ANSWER 29 OF 29 MEDLINE on STN
AN 85007250 MEDLINE
DN PubMed ID: 6480743
TI Isolation of thrombospondin released from thrombin-stimulated human platelets by fast protein liquid chromatography on an anion-exchange Mono-Q column.
AU Clezardin P; McGregor J L; Manach M; Robert F; Dechavanne M; Clemetson K J
SO Journal of chromatography, (1984 Jul 27) 296 249-56.
Journal code: 0427043. ISSN: 0021-9673.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198411
ED Entered STN: 19900320
Last Updated on STN: 19980206
Entered Medline: 19841114
AB Thrombospondin, a glycoprotein found in human platelet alpha granules, is thought to play a major role in platelet haemostatic functions. A rapid method to isolate thrombospondin for functional and structural studies was developed. Freshly prepared supernatants from thrombin-stimulated platelets were separated on an anion-exchange Mono-Q column on a fast protein liquid chromatography system. **Detection** of thrombospondin in the eluted peaks was performed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis combined with silver staining and a solid-phase radioimmunoassay with monoclonal **antibodies** directed against **thrombospondin** and other platelet granule glycoproteins. Thrombospondin was isolated rapidly to a high degree of purity using the fast protein liquid chromatography Mono-Q system (20 min), compared with the time taken with other techniques.

Short Communication

Immunohistochemical Identification of Thrombospondin in Normal Human Brain and in Alzheimer's Disease

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National Institutes of Health, Bethesda, Maryland; and
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Thrombospondin is part of a family of adhesive glycoproteins and is involved in a number of physiologic processes such as angiogenesis and neurite outgrowth. Immunohistochemical localization of thrombospondin in normal human brains was investigated in the hippocampus and inferior temporal cortex. Two antibodies (one polyclonal and one monoclonal) against thrombospondin-labeled microvesicles, glial cells, and a subpopulation of pyramidal neurons. The distribution of thrombospondin staining in patients with Alzheimer's disease was found to be comparable to control subjects. However, in patients with Alzheimer's disease a subset of pyramidal neurons that may be vulnerable in Alzheimer's disease exhibited decreased staining. This decrease in the intensity of labeling might constitute a marker for a neuronal population prone to early degeneration. In addition, thrombospondin staining was demonstrated in senile plaques in Alzheimer's disease. These results suggest that thrombospondin may be involved in the process of neuronal degeneration and senile plaque formation. (Am J Pathol 1992, 141:783-788)

Thrombospondin (TSP) is a large, multifunctional glycoprotein, and part of a family of adhesive proteins generated by alternative splicing and gene duplication.¹ TSP interacts with certain components of the cell surface and the extracellular matrix such as sulfated glycolipids, gly-

cosaminoglycans (GAGs), heparan sulfate proteoglycan (HSPG), fibrinogen, fibronectin, collagen, histidine-rich glycoproteins, and plasminogen.^{1,2} Thrombospondin promotes cell adhesion and migration and modulates responses of several cell types to growth factors.^{1,2} Through its ability to modulate endothelial cell adhesion, motility, and growth, it is a potential angiogenesis regulatory factor.^{3,4} One of the cell surface ligands for thrombospondin is HSPG, which may mediate some of the biological effects of thrombospondin on cells.⁵⁻⁷ Heparan sulfate proteoglycan produced by tumor cells of neuroectodermal origin has also been shown to bind to TSP with high affinity.⁸ Thrombospondin is found in cultured human brain glial cells⁹ and is involved in the migration of cerebellar granule cells,¹⁰ and neural crest cells.¹¹ Thrombospondin is also present in the embryonic retina, and promotes retinal neurite outgrowth,¹² making it a likely regulator of axonal growth in the embryonic nervous system.¹³

The biochemical and molecular analysis of the characteristic lesions of Alzheimer's disease (AD), neurofibrillary tangles (NFT), and senile plaques (SP), demonstrated that these pathologic changes contain a variety of abnormal elements. Neurofibrillary tangles have been shown to contain altered cytoskeletal proteins.^{14,15} Within SP and congophilic angiopathy, the main component described is the β -protein or A4 peptide (β PA4).¹⁶ Other components have also been detected such as α_1 -antichymotrypsin,¹⁷ laminin,¹⁸ and GAG and/or proteoglycans (PG).^{19,20}

To further investigate the possible events leading to the formation of the typical lesions of AD, we used antibodies against TSP, a heparin-binding protein, to exam-

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ine its distribution in the cerebral cortex of demented and neurologically normal elderly patients. The antibodies used in this study were developed against TSP1, a product of the first described gene.²¹ We do not have any evidence that they react with TSP2, a product of the second gene.²² However, in the mouse, both TSP1 and TSP2 are expressed in fetal and adult brain.²³ It is not known if both genes are expressed in human brain. If they are expressed, both TSP proteins may be recognized with the polyclonal antibody because they have many consensus amino-acid sequences. Because monoclonal antibody A6.1 recognized the same elements as the polyclonal antibody in our materials, both TSP proteins may be detected with A6.1. We observed that TSP is found in the normal human brain. In AD, it is present within SP and in a particular neuronal subpopulation that is likely to be vulnerable in the degenerative process.

Materials and Methods

The brains of five patients with no history of neurologic or psychiatric disorders (72.8 ± 6.0 years, postmortem delay less than 6 hours), and of nine patients with senile dementia of the Alzheimer type (80.8 ± 2.1 years postmortem delay less than 6 hours) were obtained at autopsy. The diagnosis of possible AD was established clinically according to NINCDS-ADRDA criteria²⁴ and confirmed neuropathologically by the presence of high densities of SP and NFT in the hippocampus and the neocortex. The brains were processed as described by Hof et al.²⁵

Forty micrometer thick sections were pretreated with 3% H_2O_2 in methanol (1:3, V:V) to eliminate the endogenous peroxidase activity. Then, they were incubated overnight at 4°C with either a polyclonal²⁶ (used at a working dilution of 1:1000) or the monoclonal A6.1²⁷ (used at a working dilution of 3.0 to 3.5 $\mu\text{g}/\text{ml}$) antibodies against a segment of TSP designated as type II, calcium dependent. After incubation, the sections were processed with an avidin-biotin kit (Vector Laboratories, Burlingame, CA), and diaminobenzidine, and then intensified by serial baths of 0.005% osmium tetroxide, 0.5% thiocarbohydrazine, and 0.005% osmium tetroxide.²⁵ Some TSP-labeled sections were counterstained with a 1% aqueous thioflavine S solution to simultaneously identify NFT and SP.

Rabbit polyclonal anti-TSP antiserum was prepared by immunizing rabbits with purified human platelet TSP. Its specificity has been demonstrated previously.²⁶ The mouse monoclonal antibody A6.1, which recognizes a calcium-dependent epitope in the 70 kda core of thrombospondin,²⁷ was provided by Dr. William Frazier, Wash-

ington University, St Louis. The antibody was purified by protein A affinity chromatography.

Results

In the cortex of control cases, the polyclonal antibody to TSP stained a subpopulation of pyramidal neurons (Figure 1A). Similar neurons were also stained with the monoclonal antibody A6.1 (Figures 1B, 2A-B). Thrombospondin-immunoreactive neurons were found in the pyramidal

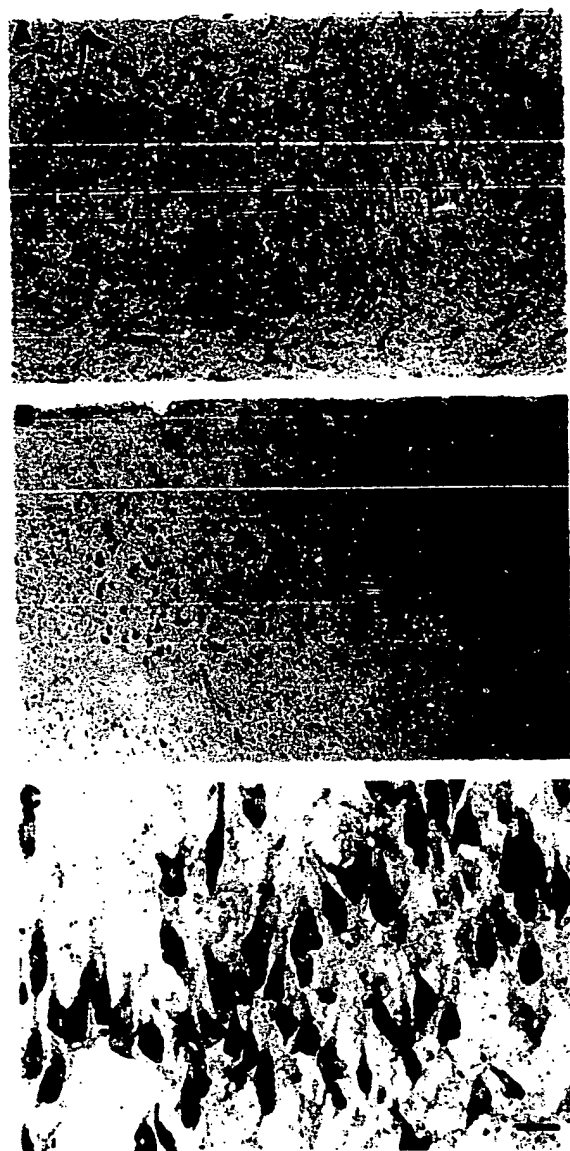


Figure 1. A: Pyramidal neurons and microvessel staining using a polyclonal antibody against TSP in a control case. B: Layer II of the entorhinal cortex in a control case stained with the monoclonal antibody A6.1; note the neuronal labeling. Magnification bar = 100 μm . C: High magnification of CA3 pyramidal neurons stained with the monoclonal antibody A6.1. Note the dark granular staining. Magnification bar = 25 μm .

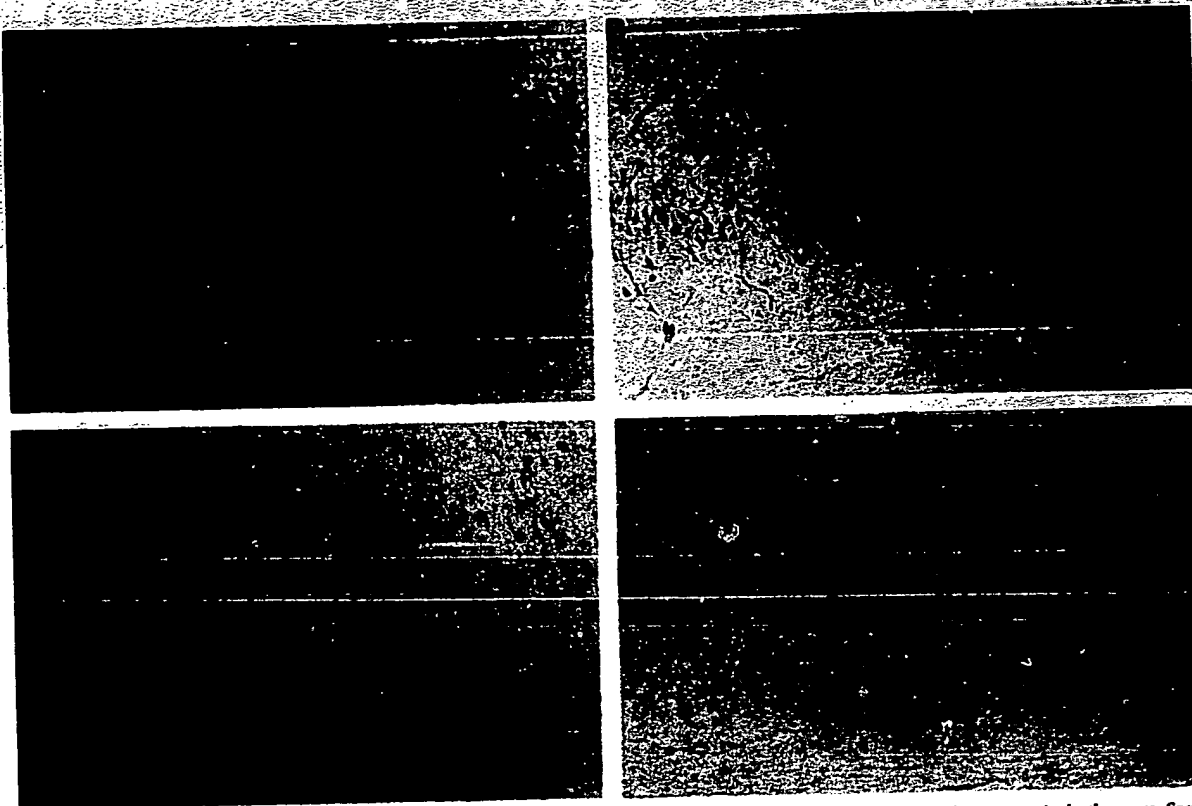


Figure 2. A: CA1 field in a control case. B: CA3 field in a control case. Note the dark staining of pyramidal neurons in both areas. Some microvessels are also labeled. C: CA1 field in AD patient; the neuronal staining is decreased, whereas a few microvessels are labeled. D: CA3 field in AD patient; the neuronal staining is decreased. Materials were stained with the monoclonal antibody A6.1. Magnification bar = 100 μ m.

layer of the CA1-CA4 fields (Figure 2A-B) of the Ammon's horn and in the large polymorphic neurons of the hilus of the dentatus gyrus. In the entorhinal cortex, layer II demonstrated the darkest neuronal staining (Figure 1B), followed by layer III, and finally layers V and VI. In the inferior temporal cortex, pyramidal neurons of the layers III and V were also darkly labeled. At higher magnification, the labeling pattern of the pyramidal neurons appeared to be finely granular (Figure 1C). With the monoclonal antibody, the neuronal staining was much stronger than with the polyclonal antibody. Glial cells were also labeled, especially in the white matter surrounding blood vessels, as well as diffuse elements in the neuropil (not shown). Staining of glial cells was weaker than neuronal staining. A few microvessels were stained with both antibodies.

The neuronal staining was markedly decreased in all AD cases as compared with control cases. For instance, using antibody A6.1, pyramidal neurons were darkly stained in control cases in CA1 and CA3 fields (Figure 2A-B), whereas in AD patients, neuronal staining was almost absent in CA1 (Figure 2C) and severely decreased in CA3 (Figure 2D). Similar decreases in staining intensity in AD brains were observed in subiculum and in layers III and V of the inferior temporal cortex. In AD

cases, no TSP staining of pyramidal neurons was observed in the entorhinal cortex. Staining of glial cells and fibrillar formations was comparable to that observed in control cases. Microvascular staining appeared to be weaker in AD as compared with control cases.

In AD, SP consistently stained for TSP and were observed in all cortical areas investigated. Both monoclonal and polyclonal antibodies against TSP-labeled SP (respectively Figure 3A-B). As demonstrated by double labeling, SP stained by thioflavine S was also labeled by anti-TSP antibodies (Figure 3B-C). The staining pattern of some SP also suggested that TSP may accumulate in dystrophic neurites surrounding the plaques.

Discussion

Our observations suggest that a subpopulation of TSP-immunoreactive neurons that appear to be vulnerable in AD is decreased or lost. This conclusion is based on the fact that weakly stained TSP-immunoreactive neurons or their absence in AD are found in the areas where neuron loss preferentially occurs in AD.^{25,28,29} Furthermore, comparable glial and vascular staining was observed in control subjects as well as AD patients.

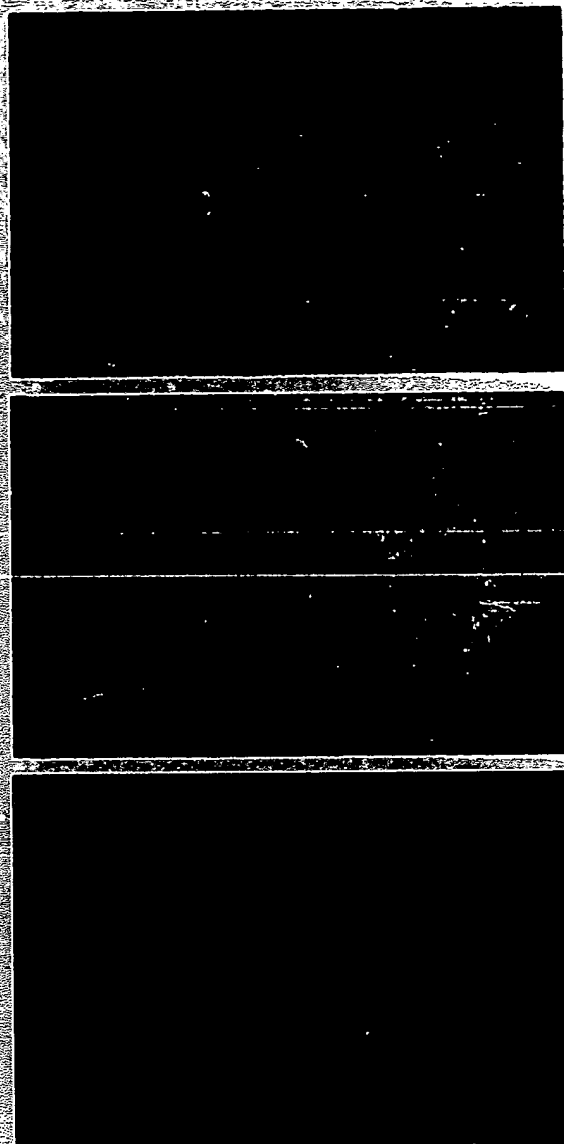


Figure 3. A: Using the polyclonal antibody against TSP, SP (arrow) and microvessels are stained in AD cases. B, C: Double labeling of amyloid deposits using the monoclonal antibody A6.1 against TSP (B) and thioflavine S under fluorescence lighting conditions (C). Note that all SP were stained by both thioflavine S and the antibody against TSP (arrowheads). Magnification bar = 100 μ m.

The presence of TSP in the central nervous system is compatible with immunochemical and biochemical analyses of human glial cells in culture that have demonstrated that these cells are able to synthesize TSP.⁹ Thrombospondin staining was noted in glial cells in normal brain⁹ but has not been previously reported in neuronal cells. We observed staining of glial cells in the brains of both control and AD cases. Furthermore, TSP has been shown to be involved in a number of physiologic processes in primary cell cultures of embryonic neurons,¹³ or in the developing nervous system.¹⁰⁻¹² It

may play a unique role in development by creating local foci of controlled balance between proteases and protease inhibitors necessary for migration and neurite outgrowth. TSP may have a similar function in the normal adult brain.

In AD cases, the granular TSP staining of pyramidal neurons appears decreased compared with control subjects. Since it is likely that the antibodies used in the present study crossreact with both TSP gene products, neuronal labeling may be related to the presence of neuron-specific TSP, which has not yet been characterized. This granular pattern could be intracytoplasmic and represent the labeling of vesicles, or nuclear receptors. It could also represent staining of surface receptors as observed in fibroblasts.³⁰ Thus, the loss of neuronal TSP staining in AD may have two explanations. Since neurons have at least two classes of TSP receptors,¹² it may simply reflect the loss of surface receptors, or it could result from the loss of these particular TSP-immunoreactive neurons that may contain a neuron-specific TSP. Pyramidal neurons are lost in AD.^{25,28,29} Furthermore, in AD, this granular TSP staining was better preserved in CA3 than in CA1. These data are suggestive of a regional pattern of vulnerability. In this respect, CA1 contains more NFT than CA3 in AD. Layer II of the entorhinal cortex did not show any TSP staining in AD whereas in control subjects, staining of pyramidal cells was common. These neurons located within layer II of the entorhinal cortex form the perforant pathway that project to the gyrus dentatus. This circuit has been shown to be severely affected in AD.³¹ Thus, the pattern of TSP-immunoreactive pyramidal cell loss parallels the neuronal degeneration and cell loss observed in AD.^{25,28,29} Interestingly, the TSP neuronal subpopulation affected in AD appears to be comparable to a population of pyramidal neurons that contain high density of non-phosphorylated neurofilament protein.²⁵ However, double-labeling studies will be necessary to determine the degree to which TSP-positive pyramidal cells also contain high levels of non-phosphorylated neurofilament protein.

A number of extracellular components such as GAG/
PG,¹⁹ laminin,¹⁸ or protease inhibitors¹⁷ are found in SP. The presence of TSP, as well as these other proteins in SP may result from vascular and/or neuronal degeneration and participation in formation of SP. Biological effects of TSP are diverse and can stimulate or inhibit proliferation depending on the cell type. However, in the central nervous system, thrombospondin appears to be expressed at sites of proliferation or migration.¹⁰⁻¹³ Since synaptic loss is one of the early events in AD pathology,³² TSP may accumulate in these areas of synaptic loss to promote regeneration of injured cells. This may explain the fact that TSP staining of SP appears to be primarily in dystrophic neurites and also that TSP neuronal staining is

decreased. In addition, as previously demonstrated, microvasculature changes are found in AD.^{20,33} TSP also plays a role in the regulation of the perivascular extracellular matrix and is an inhibitor of the angiogenesis.³ There is evidence that components of the extracellular matrix are involved in SP.¹⁸ Thus, the presence of TSP within SP may also result from vascular pathology. Biochemically, the presence of TSP within SP could reflect its high affinity for HSPG⁹ that is always found to coexist with β PA4 in SP in AD.^{18,20}

In conclusion, TSP is found in normal human brain, especially in a subpopulation of pyramidal neurons. Its decreased staining or its loss in AD might be a neuronal marker of early neuronal degeneration. These data suggest that pathologic changes linked to AD may involve a loss of specific chemically identifiable neuronal populations. Finally, TSP is found in SP and may be involved in a number of mechanisms during regeneration that may lead to formation of SP.

Acknowledgments

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BETA-AMYLOID PROTEIN INDUCES PLATELET AGGREGATION
AND SUPPORTS PLATELET ADHESION

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The amyloid precursor protein (APP) is found in many cells including neurons, endothelial cells and blood platelets. Beta-amyloid protein (BAP) is derived from APP and is deposited in brain and in cerebral microvasculature of individuals with Alzheimer's disease. In this study we demonstrate that BAP interacts with human blood platelets. We found that human BAP peptide (1-40) fibrils aggregate platelets and support their adhesion, and these interactions are mediated through platelet membrane integrin receptors.

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Beta-amyloid protein (BAP, 4kD) is released by the alternative processing of amyloid protein precursor (APP). Several different mechanisms (1) have been proposed which could lead to the generation of BAP, which accumulates by amyloid fibril polymerization in the brain and blood vessels in Alzheimer's disease (AD), Down's syndrome and aged normal people. Platelets contain concentrated APP in α -granules (2,3), and as a minor species associated with platelet membranes which may be shed during platelet activation. (4). Human platelets activated by thrombin release APP and prostacyclin suppresses this reaction (4). Some forms of platelet APP contain Kunitz-protease inhibitor (KPI) domain (5). Low amounts of APP of similar size to those released by the platelets occur in plasma (6,7). Stimulated platelets release membrane fragments containing full-length C-terminal and N-terminal immunoreactive APP which should contain the intact BAP (4kD) sequence. The platelet released APP may therefore contribute to the formation of circulating β amyloid protein, and may be a source of BAP in amyloid deposits (8). Several roles for APP have been postulated (1). It is possible that forms released from platelets have a growth factor-related function. It is also likely that through its KPI regions APP may participate in the coagulation cascade by inhibition of factors IXa and XIa. A third possible role for platelet APP is to modulate cell-cell contact. Recent study of thrombosis prone patients (9) provided evidence of the association of APP and platelet-derived microparticles. APP was also found in chronic thromboemboli (10). Immunohistochemical analysis indicated colocalization of APP antigen with platelet glycoprotein GPIIb/IIIa in fibrin-rich thrombi and in a single layer of endothelial cells.

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Thus a physiological role for secreted APP in clot formation is likely. In this study we explore the interaction of BAP fibrils including full length peptide AP1-40 (APP597-636 based on APP of 695 residues) and its extracellular domain AP1-28 (APP597-624). Preliminary data are presented on a possible mechanism of interaction of BAP fibrils with platelets which may play a role in both thrombosis and in Alzheimer's disease.

METHODS

Platelets isolation: Platelets were isolated by differential centrifugation. Platelet-rich-plasma (PRP) was prepared by centrifugation of the citrated blood at room temperature for 20 min at 150xg. Platelets were pelleted by centrifugation of PRP at room temperature for 20 min at 1,000xg. The platelet pellet was resuspended in Hepes buffer, pH 6.5 (Hepes-buffered Tyrode's solution containing 5mM Hepes, 4mM NaH₂PO₄, 137mM NaCl, 2.6mM KCl, 1 mM MgCl₂, 5mM glucose and 1 mg/ml albumin) containing 5 U/ml apyrase and sedimented by centrifugation. Finally, platelets were suspended in Hepes buffer, pH 7.5 at 3×10^8 platelets/ml. For the serotonin secretion study, platelets in PRP were loaded with ³H-hydroxytryptamine [³H-HT] and washed as described above.

Formation of BAP fibrils: Two synthetic peptides (Bachem, California) were used in this studies: AP1-40 (APP597-636) and AP1-28 (APP597-624), full length BAP and its extracellular domain, respectively. Peptides were suspended to the concentration of 5 mg/ml in deionized water. Fibrils were formed by incubation of peptides overnight at 37°C in Ca²⁺, Mg²⁺-free PBS at 100-1000 µg/ml before use.

Platelet aggregation and secretion: 400 µl of platelets (3×10^8 pl/ml) were placed in an aggregometer cuvette and incubated with stirring at 37°C for 3 min in the presence or absence of 200 µg/ml of fibrinogen. Agonist was then added and incubation was continued for 3 min. For measurements of secretion, stopping solution containing 2% formaldehyde and 5 mM EDTA was added and platelets centrifuged for 5 min. Radioactivity in supernatant was counted in a β-scintillation counter. Aggregation is presented as a percent of light transmission and secretion as a percent of total [³H-HT] taken up by platelets.

Platelet adhesion assay: BAP peptides were diluted to 100 µg/ml in Ca²⁺, Mg²⁺-free PBS (Sigma) and 100 µl of this solution were incubated on 96-wells microtiter plates (Costar) overnight at 37°C and blocked with 1% BSA for 1 hr at room temperature (RT). The wells were then washed and 100 µl of platelet suspension in the Tyrode's buffer were added. Platelets were incubated in the wells for 60 min at RT and nonadherent platelets were removed. Wells were then washed three times with 200 µl of Tyrode's buffer. The number of adherent platelets was determined by BCA protein assay (Pierce), as described earlier (11).

Binding of various proteins to BAP peptide fibrils. Binding of fibrinogen, thrombospondin and purified GPIIb/IIIa was performed in solid-phase assay. 96-wells microtiter plates were coated with BAP peptides as described above, blocked with 1% albumin and various proteins added for 1 hr at 20°C. Wells were rinsed 3 times in PBS and bound biotinylated fibrinogen or GPIIb/IIIa detected by binding of streptavidin linked with horseradish peroxidase. In the second system monoclonal antibody against thrombospondin or GPIIb/IIIa was followed by the addition of alkaline phosphatase conjugated goat-anti mouse antibody. Color was developed after addition of appropriate substrate and optical density at 405 nm was measured. In both systems optical density is proportional to the amount of bound protein.

RESULTS

Aggregation of platelets induced by BAP fibrils.

Synthetic AP1-28 and AP1-40 peptides form fibrils in vitro that are similar in ultrastructure to the in vivo amyloid peptides fibrils (12). We observed that fibrils formed by peptide AP1-40

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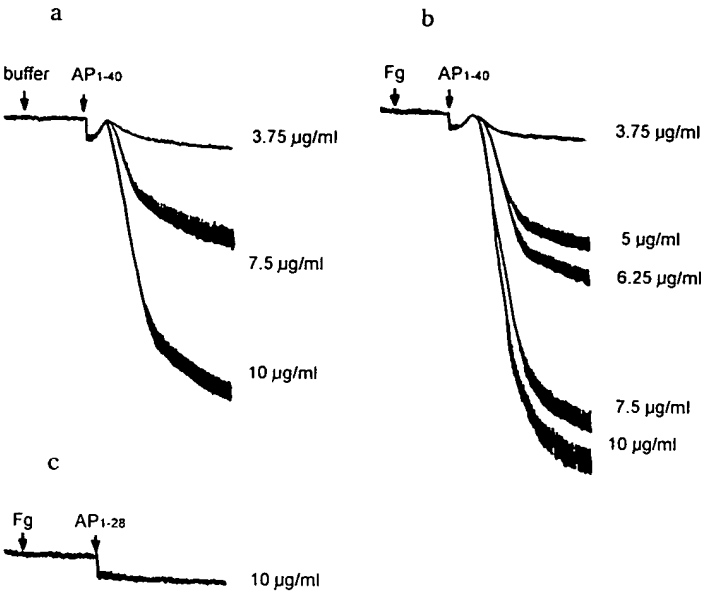


Fig.1. Aggregation of human blood platelets by AP 1-40 peptide. Platelets (3×10⁸/ml) were incubated with stirring at 37°C with indicated concentrations of AP 1-40 peptide fibrils in the absence (a) or presence of 200 µg/ml of fibrinogen (b). Addition of AP1-28 fibrils to platelet suspension did not have any effect on aggregation of platelets (c).

(Fig 1ab) induced platelet aggregation in a dose dependent manner with EC₅₀ ~ 8 µg/ml without and 6.5 µg/ml with external fibrinogen added, respectively. Platelet aggregation by βAP fibrils was similar to collagen induced platelets aggregation with respect to delay after addition of fibrils and the start of shape change. Stimulation of platelets by AP1-40 fibrils induced the release of dense granules as measured by the release of ³H-serotonin (Table I). In contrast AP1-28 fibrils at the concentration up to 125 µg/ml (Fig 1c) did not promote aggregation and dense granule

Table I		
	Aggregation	Release (³ H-HT)
AP 1-40 (no fg)	44%	26%
AP 1-40 (+ fg)	70%	35%
AP 1-40 (+ GRGDS)	10%	21%
AP 1-40 (+ ASA)	20%	0%
AP 1-28 (+ fg)	0%	3%
ADP (+ fg)	44%	26%
ADP (+ GRGDS)	0%	2.6%
ADP (+ ASA)	10%	0%
Thrombin	75%	80%
Control	0%	1.3%

secretion. Aggregation but not secretion induced by AP1-40 fibrils (10 $\mu\text{g/ml}$, 2.3 μM) was inhibited by GRGDSP peptide (100 μM). In contrast, an 11 amino acid peptide AP1-11 (APP597-617) containing an RHDS sequence, or GRGESp up to the concentration of 100 μM had no effect on AP1-40 induced platelet aggregation. Addition of aspirin (200 μM) blocked completely platelet secretion and the second wave of aggregation whereas 1 μM of PGE₁ blocked both aggregation and secretion promoted by AP1-40 fibrils.

Adhesion of platelets to BAP fibrils.

We also studied adhesion of human blood platelets to BAP fibrils immobilized on plastic microtiter wells. Platelets adhere to both AP1-40 and AP1-28 coated wells, similar number of platelets adhere to fibronectin coated plates, while no platelets adhered to BSA (Table II). Platelets preincubated with 1 μM PGE₁ adhered to AP1-40, however they failed to adhere to the AP1-28 coated wells (Table II). Adhesion of platelets to AP1-40 was time and divalent cation dependent (Fig 2a). In the presence of EDTA and EGTA platelet adhesion to AP1-40 was inhibited by 82% and 80%, respectively.

Next, the possible receptors involved in adhesion of platelets to AP1-40 peptide were examined. Platelets were preincubated with various antibodies and peptides for 10 min at room temperatures and then allowed to adhere to AP1-40 fibrils (Fig 2b,c). Monoclonal antibody against the platelet GPIIb/IIIa receptor (A2A9, donated by Dr. J. Bennett, University of Pennsylvania) at 20 $\mu\text{g/ml}$ inhibited adhesion of platelets to AP1-40 by 80% (Fig 2b). Also thrombasthenic platelets, which possess only 10% of the normal GPIIb/IIIa population, showed impaired adhesion to AP1-40 peptide (25% of control, data not shown). Antibody against platelet GPIa/IIa (clone Gi9, AMAC) inhibited platelet adhesion to AP1-40 by 50%. We also observed (Fig 2c) the inhibitory effect of the GRGDSP peptide (100 μM) as well as RGD containing peptide echistatin, a snake venom derived disintegrin (5 μM , data not shown). In contrast AP1-11, which contains RHDS sequence and GRGESp or SFLLRN peptides at the concentration of 100 μM , did not have any effect on the adhesion of platelets to AP1-40 (Fig.2c).

Binding of GPIIb/IIIa, Fg and TSP to BAP fibrils.

In order to test if AP1-40 interacts directly with GPIIb/IIIa we studied the binding of purified GPIIb/IIIa to AP1-40 fibrils. We did not detect direct binding of GPIIb/IIIa to A1-40 (Fig. 3a) in solid-phase binding system. The lack of AP1-40 - GPIIb/IIIa interaction was independent of the binding system used. In the first system binding of purified GPIIb/IIIa to plates coated with A1-40

Table II

	Adhesion of platelets (O.D. at 595 nm \pm SD)	
	- PGE ₁	PGE ₁
A1-40	0.662 \pm 0.027	0.533 \pm 0.009
A1-28	0.305 \pm 0.072	0.059 \pm 0.013
Fibronectin	0.589 \pm 0.009	0.198 \pm 0.008
Albumin	0.086 \pm 0.020	0.010 \pm 0.001

Data presented are the mean of at least three different studies.

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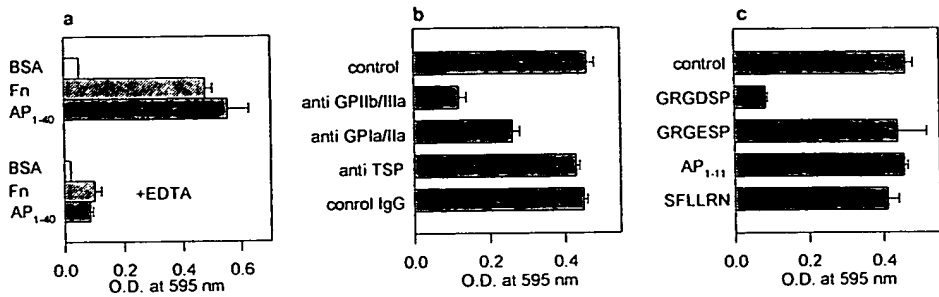


Fig. 2.
a) Platelet adhesion to A1-40 [cross-hatched bars], fibronectin [diagonally hatched bars] or albumin [open bars] in the presence of PGE1. Adhesion to all the substrates was inhibited by EDTA (4mM).
b) Platelet adhesion to the peptide A1-40 in the presence of PGE1 and various monoclonal antibodies. All antibodies were used at 20 μ g/ml. Antibody against GPIIb/IIIa inhibited platelet adhesion in about 80% and antibody against GPIa/IIa in about 50%. Control or anti-TSP antibody had no effect.
c) Platelet adhesion to peptide AP1-40 in the presence of various peptides (100 μ M). Platelet adhesion was inhibited by GRGDSP in about 85% but not by AP1-11, which contains RHDS sequence. Control peptide GRGESp or thrombin receptor peptide SFLLRN at the same concentration have no effect on adhesion of platelets to AP1-40.

was detected using an antibody to GPIIb/IIIa or biotinylated GPIIb/IIIa. In the second, we used 125 I-labeled A1-40 and studied its ability to bind GPIIb/IIIa immobilized on the microtiter plates. Under the same conditions AP1-40 was capable of binding fibrinogen and thrombospondin, two major platelet binding proteins. Biotinylated fibrinogen [Fg] (Fig 3b) bound to the wells coated with AP1-40 peptide but not to BSA coated wells in a concentration dependent and saturable manner. Similarly, thrombospondin [TSP] (Fig 3c) bound to AP1-40 peptide as detected using anti-thrombospondin monoclonal antibody.

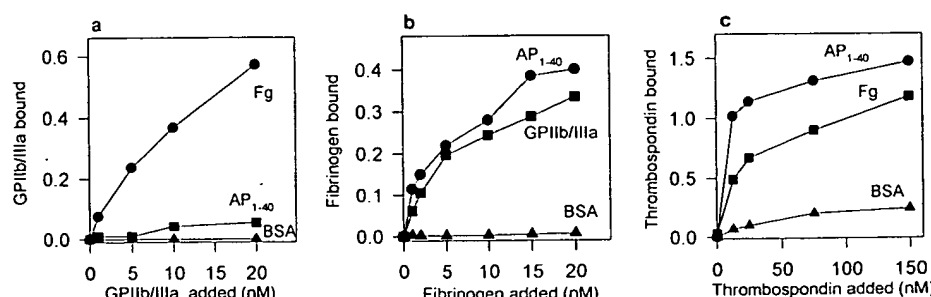


Fig. 3.
Binding of purified GPIIb/IIIa (a), fibrinogen (b), and thrombospondin (c) to AP1-40 fibrils. Plates were coated with AP1-40 peptide or control proteins as described in methods. GPIIb/IIIa, fibrinogen or thrombospondin was allowed to bind to AP1-40 fibrils for 1 hr at room temperature. Amount of bound protein was detected after addition of appropriate substrates and presented as O.D. at 405 nm.

DISCUSSION

We have shown that human β AP peptide fibrils support platelet adhesion and aggregation of washed human blood platelets. β -amyloid fibrils may interact with specific platelet receptor(s), linked to a second messenger system, which leads to platelet release reaction, expression of platelet fibrinogen receptors GPIIb/IIIa and platelet aggregation. Antibody against GPIIb/IIIa blocked the adhesion of platelets to β AP fibrils but we did not observe the direct interaction of β AP with purified GPIIb/IIIa. Therefore GPIIb/IIIa is likely to be involved in the interaction of platelets with β AP, however this interaction is not direct but possibly through released and surface bound TSP and/or fibrinogen, which bind to GPIIb/IIIa (13-15). It is also possible that other integrins like GPIa/IIa may serve as receptors for β AP. It has been shown that thrombospondin, a large adhesive protein secreted from platelets, and also found in cultured human brain glial cells (16) may also be involved in the process of neuronal degeneration and senile plaque formation (17). Therefore, TSP may be involved in the interaction of β AP with cellular integrins. A 109-amino-acid construct C109 with a sequence analogous to the C-terminal of APP was found to promote cell adhesion of the human monocytoid cell line U937 (18). This fragment as well as a synthetic peptide identical with the first 28 N-terminal extracellular residues of β AP promote adhesion of those cells through an integrin-like receptor. As shown by Schubert et al. (19) APP molecules were able to stimulate adhesion of nerve cell line PC12. PC12 cells were found to interact with laminin, collagen IV, and fibronectin with integrin-related glycoproteins involved in attachment and process outgrowth. This and our observation suggest that there may be a common mechanism of interaction between β AP and various cells and cellular integrins are likely to be involved in such interactions. The role of RGD-binding site may be important since it is present on a broad number of integrin receptors. Interaction of β AP fibrils with platelets and with fibrinogen and thrombospondin may further suggest the role of APP in cell-cell and cell-matrix adhesion.

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Structure–activity relationship of heparan sulphate

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Introduction

Heparan sulphate (HS) is the major glycosaminoglycan on mammalian cell surfaces and in basement membranes [1,2]. Cell surface HS is attached covalently to two major classes of protein, the transmembrane syndecans and the glycosylphosphatidylinositol (GPI)-anchored glycoproteins and in basement membranes the principle component that bears HS is a large multidomain protein called perlecan. These protein cores typically contain 2–3 HS chains positioned in close proximity to each other along a short stretch of peptide in which the glycanated serine residues are in sequences of short Ser-Gly repeats with hydrophobic and acidic amino acids in the vicinity of the glycanation region [3]. This clustering of HS chains is suggestive of a co-operative mode of action which may be of special relevance to the role of HS-proteoglycans in regulating cell adhesion and migration on biomatrices.

Biosynthesis and structure of HS

In common with most other glycosaminoglycans, HS is linked to core proteins by a tetrasaccharide sequence at the reducing end of the chain com-

posed of Xyl-Gal-Gal-GlcA- with the xylose in an O-glycosidic linkage to serine. The polymer is built up on this linkage sequence by the addition of GlcNAc and GlcA in alternate sequence to form polysaccharides ranging in length from 50 to 200 GlcNAc–GlcA disaccharide repeats. During polymerization enzyme-mediated modifications to the growing 'heparan' chain [4] are initiated beginning with the conversion of a proportion (normally 40–50%) of the GlcNAc units to the unique N-sulphated glucosamine (GlcNSO₃) catalysed by deacetylase/N-sulphotransferase enzymes. Further modifications include the epimerization of GlcA to the C-5 epimer iduronate (IdoA) followed by the addition of sulphate groups to C-2 of IdoA, C-6 of the amino sugars, and more rarely to C-3 of GlcNSO₃. These modifications are confined to regions of N-sulphation and are largely excluded from areas where N-acetylated disaccharides predominate. This restriction acts as a constraint on the sequence variability seen in HS [5] and leads to the formation of domains of relatively high sulphation (S-domains) and IdoA content. These S-domains arise because the primary changes in HS synthesis (i.e. conversion of GlcNAc into GlcNSO₃) are mainly directed to localized areas of the polymer chain [6,7] rather than being uniformly distributed (Figure 1). The S-domains are thus separated by relatively unmodified N-acetyl-rich regions which act as spacers giving

Abbreviations used: HS, heparan sulphate; GPI, glycosylphosphatidylinositol; IdoA, iduronate; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; AT-III, anti-thrombin III.

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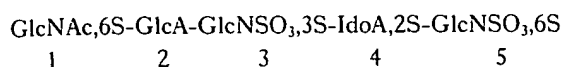
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the HS chain its distinctive character from the chemically related heparin which is made up almost entirely of N-sulphated disaccharide units [8]. For a thorough review of HS and heparin synthesis see [4].

HS messages in the sequence

Anticoagulation and polymorphism

HS plays many important roles in the biology and biochemistry of the cell. It is well recognized that HS is important in maintaining a non-thrombogenic surface on the endothelial lining of blood vessels due to the presence of a unique pentasaccharide sequence (first identified in anticoagulant heparin) of the following structure:

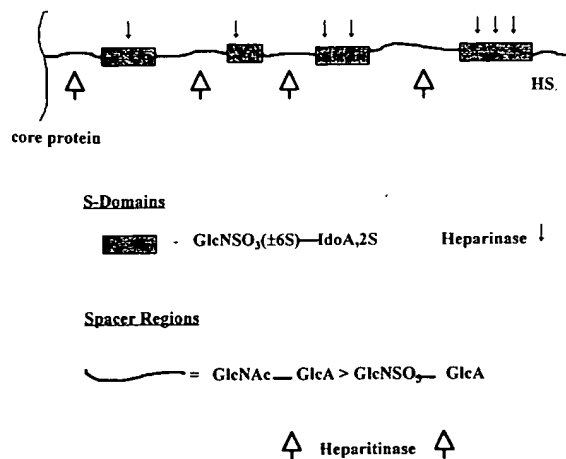


This sequence binds to anti-thrombin III (AT-III) with high affinity and greatly accelerates the rate at which it binds and inhibits

Figure 1

Domain structure of HS

HS consists of hypervariable sulphated domains (S domains) separated by regions of low sulphation (spacer regions). The S-domains contain the majority of N- and O-sulphate groups and IdoA residues, whereas N-acetylated disaccharides predominate in the spacer regions. The enzyme heparitinase (favoured substrate GlcNAc/GlcNSO₃-GlcA) can be used to release the S-domains (typical size range 2–7 disaccharides), whereas heparinase cuts HS within the S-domains at the GlcNSO₃(±6S)-IdoA,2S linkage. The above model, although giving a simplified view of HS, is applicable to the majority of HS polymers produced by different cell types. Variations in sulphation pattern, particularly involving the O-sulphate groups, are superimposed on a common S-domain organization to give rise to cell-type-specific HS species. Rat liver HS is the major exception to the spaced arrangement of S-domains; the liver HS contains on average three S-domains in close apposition in the distal region of the chain [7].



thrombin and factor Xa [9,10]. It is noteworthy that an unmodified sugar (GlcA) is present at residue 2 and the rare 3-O-sulphate is present on the GlcNSO₃ at residue 3. A GlcNAc residue at position 1 is not absolutely required (GlcNSO₃ will suffice) but it inhibits any tendency for the adjacent GlcA to be converted into IdoA; the 6-O-sulphate group on the GlcNAc is vital for AT-III binding. The AT-III-binding pentasaccharide sequence is thus brought about by the concerted action of a number of enzymes which effect an incomplete but controlled set of modifications to short sections of the HS/heparin chain. This reflects the clearly observed fact that in HS (and in heparin also) the potential for epimerization and sulphation of N-sulphated regions is never fully realized and a particularly notable feature of HS is that although its fine structure varies from one cell type to another, this variation is largely due to different levels of O-sulphation imposed on a common backbone structure of spatially discrete S-domains (Figure 1). The AT-III binding sequence may be considered as a distinct short region at the interface of an S-domain with an N-acetyl-rich spacer.

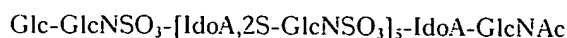
S-domains: molecular size, sulphation and growth control

The S-domains in HS vary in size from two to about seven or eight disaccharide units, although occasionally even longer domains are found. The S-domains are designed for protein recognition and their fine structure and positioning along the HS chain are important aspects of their recognition properties. Examples of the importance of S-domain spacing have come from studies of interferon γ and platelet factor 4 (dimeric and tetrameric proteins respectively) which span S-domains separated by about 10–12 disaccharide units [11,12].

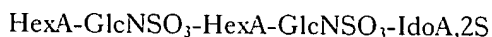
Basic and acidic fibroblast growth factor (bFGF and aFGF respectively) both require HS for their biological activity [13] and although the mode of action of HS is obscure it is likely that some form of conformational change is induced in the growth factors which enables them to bind in a productive manner to their signal-transducing receptors [14]. One popular view is that an active site on HS will bind two FGFs, which interact directly with each other to form a dimeric, receptor-binding ligand [15,16].

The detailed structure of the high-affinity binding site for bFGF in HS has been studied by several groups. There is a general consensus that

activation of bFGF requires S-domains of 6–7 N-sulphated disaccharide units with IdoA residues and 2-O-sulphate groups being major features of the binding region [17,18]. An S-domain of seven disaccharides was isolated from fibroblast HS using the enzyme heparitinase (Figure 1). The S-domain was named oligo-H, and it contained a highly regular internal repeat unit of the following sequence:



The sequence was devoid of 6-O-sulphate groups but it still bound to bFGF with a similar affinity to that of the parent HS [19]. When this sequence was first identified it was unclear whether it contained all the structural features required to promote the activity of bFGF. If it was not an activating sequence, then what was its purpose? Oligo-H was much longer than a pentasaccharide sequence proposed to be the minimal binding site for bFGF [20]; this structure contained a single IdoA,2S in the sequence:



where the HexA could be GlcA or IdoA. The presence of 6-sulphates was not required for binding of such short saccharides and this view was confirmed by studies on the crystal structures of heparin tetra- and hexa-saccharides and bFGF [21]. The N-sulphate groups and IdoA,2S in this pentasaccharide were shown to be important for binding to bFGF, but short sequences of the above type do not activate the growth factor. This may imply that the requirement for longer sequences reflects the need to bind two bFGFs in a single site as already discussed.

Studies on N-sulphated saccharides derived from heparin (i.e., IdoA,2S-GlcNSO₃,6S repeat units) and on selectively desulphated derivatives supported the view that 2-O-sulphate and N-sulphate groups were important for bFGF binding, with no obvious role for 6-sulphates [18]; however, 6-sulphate groups were needed for bFGF activation [18], and it was concluded that the active site recognized both the growth factor and its signalling receptor, with the 6-sulphates presumably being part of a subsite that bound the receptor. This idea arose because of an earlier suggestion that HS must bind the FGF receptor [22] as well as bFGF so that receptor and ligand would be brought into close proximity.

Heparins fully depleted of 6-sulphate groups were shown to be inhibitors of bFGF [18].

Because the high-affinity oligo-H sequence (also devoid of 6-sulphates) is a naturally occurring structure in fibroblast HS, it may be a negative regulator of bFGF-driven cell growth, whereas comparable sequences with the necessary complement and positioning of 6-sulphate groups should activate the growth factor. In the case of bFGF, therefore, the message in the HS sequence may be suppressive or stimulatory for growth depending on the structural characteristics of the S-domains expressed on cell surfaces.

Studies on other growth factors including aFGF [18,23,24] and hepatocyte growth factor [25] indicate that they have different structural requirements for binding to HS and both of these growth factors seem to recognize 6-sulphate groups as substituents of their binding domains. Differential binding and activation of aFGF and bFGF is observed in HS synthesized at different stages of development of the neuro-epithelium [24]. In common with bFGF, hepatocyte growth factor and aFGF also display near optimum binding to S-domains of six to seven disaccharides in length.

Summary

HS influences fundamental cellular properties and biochemical processes at the cell surface. In addition to the issues already discussed, it has a profound effect on cell adhesion and migration through its interaction with many extracellular matrix proteins, most notably fibronectin and thrombospondin; it is closely linked to lipid metabolism through its capacity to bind low-density lipoprotein and lipoprotein lipase; and aberrations in HS structure and degradation are linked to human malignancy and Alzheimer's disease [26,27]. The subtle variations in HS structure enable it to distinguish between families of related proteins such as the FGFs, the chemokines [28] and the TGF β s [29]. The multifunctional nature of HS is the result of its structural diversity and strategic positioning in the pericellular domain. The biosynthesis of HS, in common with other complex carbohydrates, is not directed by any known template yet the system is clearly subject to quite precise control so that in general, the HS family has a common domain organization that is finely tuned at the cellular level to produce HS species of variable length, fine structure and biological properties. A major challenge for future research will be to

unravel the regulatory mechanisms that determine the molecular structure of HS. It remains unclear whether these mechanisms are entirely intrinsic in nature or subject to substantial modulation by the cellular microenvironment.

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ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs) is transcriptionally induced in beta-amyloid treated rat astrocytes

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Abstract

Beta-amyloid (A β) deposition is considered to be of the most crucial events in the onset of Alzheimer's disease (AD). To identify factors involved in the exacerbation of AD, we investigated transcriptionally A β -induced genes using a cDNA subtraction technique in rat astrocytes as previously reported. One subtracted gene that showed the A β -induced expression was rat ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs). In this report, we present the deduced sequence for the mature form of rat ADAMTS-4 and demonstrate the induction of its mRNA by treatment of cells with A β . Our results suggest a degradation of the extracellular matrix occurring in the brain of AD patients and a possibly significant role of this enzyme in the progression of AD. © 2000 Published by Elsevier Science Ireland Ltd.

Keywords: Beta-amyloid; Alzheimer's disease; A disintegrin and metalloproteinase with thrombospondin motifs-4; Rat astrocyte; cDNA subtraction

Beta-amyloid (A β) is thought to be one of the most critical elements that constitute senile plaques observed in Alzheimer's disease (AD) brains. This peptide was demonstrated to possess direct toxicity to neurons and to activate astrocytes in the insoluble aggregated form that contains β -sheet structures. Its deposition in senile plaques is considered to be one of the most crucial events in the onset of this disease [5,8,17,20,26].

Numerous activated astrocytes in lesions as occurs in many other neurodegenerative disorders, is a common feature of an AD brain. The cells are found surrounding the plaques and extend their processes into the lesions [17,18]. The observation that the plaque-associated activated astrocytes show morphological changes and produce several inflammatory factors in AD [5,9,11,17,18] leads us to a possibility that the activated cells in AD lesions have some influence on neighboring neurons and contribute to an aggravation of the disease.

It is unknown whether A β itself activates astrocytes in vivo, but there is little doubt that the association of this molecule with astrocytes is of great significance in the

lesions of AD brains. The contribution of A β to the progression of neuropathologic changes mediated by astrocytes and the actual influence of glias on neurons in AD brains have not been well clarified. Thus, it remains important to investigate the expression profiles of astrocyte-derived factors that show transcriptionally altered changes by A β treatment, in an effort to identify key molecules in AD brains that may be intimately associated with disease progression.

For the purpose of investigating transcriptionally regulated factors that may be implicated in AD progression, we searched for A β -induced cDNAs derived from rat astrocytes by cDNA subtraction as previously described [21]. The cultured cells, more than 90% of which were positive for glial fibrillary acidic protein by immunofluorescence staining, were prepared from cerebral cortex and hippocampus of Wistar rat fetuses using a previously described method [13]. One subtracted cDNA fragment that showed A β -induced expression of original mRNA (Fig. 2) was a novel gene. This fragment (344 bp) has 84.8% nucleotide homology with human ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs) mRNA and includes the stop codon and each part of the coding region and the 3'-untranslated region (UTR). ADAMTS-4 has also been referred to as aggrecanase-1 [24] and its

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Metalloproteinase domain
FASLSRFVET LVVADDKMAA FHGAGLKHYL LTVMAAAAKA FKHPSIRNPV NLVVTRLVIL 60
*****
FASLSRFVET LVVADDKMAA FHGAGLKRYL LTVMAAAAKA FKHPSIRNPV SLVVTRLVIL 60

GSGQEVPQVG PSAAQTLRSF CTWQKGLNPP NDSDPDHFD T AILFTRQDLC GVSTCDALGM 120
***.* **** *..*..*..* ..*..*..*..* ..*..*..*..* ..*..*..*..*
GSGEGEPQVG PSAAQTLRSF CAWQKGLNTP EDSDPDHFD T AILFTRQDLC GVSTCDTLGM 120

AGVGTVCDDPA RSCAIVEDDG LQSAFTA AHE LGHVFNMLHD NSKPCANLNG QGSSSRHVMA 180
*..***** ..*..***** ..*..***** ..*..***** ..*..***** ..*..*****
ADVGTVCDDPA RSCAIVEDDG LQSAFTA AHE LGHVFNMLHD NSKPCISLNG PLSTSRHVMA 180

PVMahVDPEE PWSPCSARFI TDFLDNGYGH CLLDKPEAPL HLPVTFPGKD YDADRQCQLT 240
*****
PVMahVDPEE PWSPCSARFI TDFLDNGYGH CLLDKPEAPL HLPVTFPGKD YDADRQCQLT 240

FGPDSSHCPO LPPPCAALWC FGHNLGHAMC QTKHSPWADG TPCGPAQACM GGRCLHVDQL 300
*****
FGPDSRHCPQ LPPPCAALWC SGHNLGHAMC QTKHSPWADG TPCGPAQACM GGRCLHMDQL 300

Tsp Type 1 motif
KDFNIPQAGG WGPWGPWGDC SRTCGGGVQF SSRDCTKPVP RGGKGYCEGR RTPFRSCNTK 360
*****
QDFNIPQAGG WGPWGPWGDC SRTCGGGVQF SSRDCTRPVP RGGKGYCEGR RTRFRSCNTE 360

NCPHGSALT F REEQCAAYNH RTDLFKSFPG PMDWVPRYT G VAPRDQCKLT CQARALGYYY 420
*..***** ..*..***** ..*..***** ..*..***** ..*..***** ..*..*****
DCPTGSALT F REEQCAAYNH RTDLFKSFPG PMDWVPRYT G VAPQDQCKLT CQARALGYYY 420

VLEPRVADGT PCSPDSSSVC VQGRCIHAGC DRIIGSKKKF DKCMVCGGNG SSCSKQSGSF 480
*****
VLEPRVVDGT PCSPDSSSVC VQGRCIHAGC DRIIGSKKKF DKCMVCGGDG SGCSKQSGSF 480

KKFRYGSDV VTIPAGRTHI LVRQQGSGSL KSIYLALKLA DGSYALNGEY TLMPSSTDV 540
*****
RKFRYGYNNV VTIPAGATHI LVRQQGNPGH RSIYLALKLP DGSYALNGEY TLMPSSTDV 540

LPGAVSLRYS GRTAASETLS GHGPLAQPLT LQVLVAGNPQ NVRLRYSFFV PRPVPSTPRP 600
*****
LPGAVSLRYS GATAASETLS GHGPLAQPLT LQVLVAGNPQ DTRLRYSFFV PRPTPSTPRP 600

PPQNLQRRR EILEILRKRT WAGRK 625
*..*..*..* ..*..*..* ..*..*..*
TPQDLHRRR QILEILRRRP WAGRK 625

```

Fig. 1. Comparison of the mature form of ADAMTS-4 amino acid sequences. The amino acid residues for rat (top line) and human (bottom line) ADAMTS-4 are shown and have 92.3% homology, based on identical matches. Domains are denoted above the sequences by arrows. Zinc-binding motif is underlined.

sequence was first determined from size-fractionated human brain cDNA libraries [12]. Based on the sequence of the 'mature form' (a form that excludes the propeptide region after cleavage by furin, a serine protease responsible for processing of proproteins [19]) of human ADAMTS-4, we determined the sequence of this rat counterpart (rat ADAMTS-4) by polymerase chain reaction (PCR) (registered in DDBJ/EMBL/GenBank with the accession number AB042271, AB042272, AB042273). The mature form of rat ADAMTS-4 has 625 amino acids and shares a 92.3% amino acid homology (87.7% nucleotide homology, data not shown) with the human protein (Fig. 1). The required sequence for furin cleavage in rat ADAMTS-4 revealed -R-R-T-K-R¹-F-A-S-.

Aggrecan is the major and an essential proteoglycan of cartilage and is responsible for its compressibility and stiffness. The core protein of aggrecan, covalently binds to extensive glycosaminoglycans (GAGs), hyaluronic acid and link proteins to construct large aggregates, forming

constituents of the extracellular matrix (ECM). This proteoglycan is also distributed in brain under the tissue-specific regulation on sulfate content and localization on the same GAG chains of the core protein [22].

Aggrecanase is responsible for cleavage at the Glu373-Ala374 bond of aggrecan core protein while matrix metalloproteinases (MMPs) clip between Asn341 and Phe342, and the increase in aggrecanase activity is thought to play a key role in cartilage damage (see references in [24]). ADAMTS-4 (aggrecanase-1) has been purified from IL-1 stimulated bovine nasal cartilage conditioned media and shown to belong to a new protease family ADAMTS [24]. ADAMTS lack a transmembrane domain and contain carboxy thrombospondin motifs that are thought to be responsible for binding to GAGs that constitute the ECM [15]. In fact, a majority of ADAMTS-1 was shown to be associated with the ECM [14]. It is likely that this motif may also function in binding the enzyme to its substrate aggrecan for a quick remodeling of the ECM.

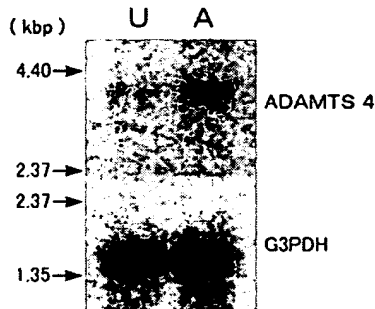


Fig. 2. Northern blot analysis of ADAMTS-4. Each lane contains 2 μ g of poly (A)+ RNA isolated from untreated (U) and beta-amyloid (25 μ M) treated (A) rat astrocytes. The blot was hybridized with the random primed subtracted ADAMTS-4 cDNA probe, and exposed for 16 h. As an internal control, the blot was hybridized with G3PDH cDNA control probe (CLONTECH) and exposed for 3 h as shown.

Expression of rat aggrecanase-1 mRNA was induced in rat cultured astrocytes that were treated with A β for 15 h. As shown in Fig. 2, the A β -induced level was about 8.1-fold higher when compared with untreated cells, as quantified by the intensity of radioactive bands on a northern blot and normalized to those of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH, used as internal control; Fuji Film, BAS2000). The size of rat ADAMTS-4

mRNA was about 3.7 kb, which is smaller than its human counterpart that is reported to be 4.3 kb [1]. This size discrepancy between the rat and human transcripts appears to result from variations in the untranslated region or the propeptide region, because the numbers of nucleotides in the processed ADAMTS-4 region of both forms is identical. Next we determined the tissue expression profile of rat ADAMTS-4. Northern blots of poly (A)+ RNA extracted from rat tissues (CLONTECH) were hybridized with random primed rat ADAMTS-4 cDNA probe (Fig. 3). The transcript of rat ADAMTS-4 was detectable only in brain, as the 3.7-kb transcript, similar to the result from rat astrocytes mRNA in Fig. 2. The ADAMTS-4 expression seen in human lung and heart were not detectable in rat. Clearly, this gene is inducible by inflammatory stimuli (Fig. 2, lane A). Hence, other tissues from brain could also express this gene when neuroinflammatory phenomena occur and there is a possibility that at steady state rat ADAMTS-4 expression in heart and lung is weaker than its human counterparts.

In another study, although aggrecanase activity was shown to be increased by IL-1 treatment [1,10,24], it was not entirely clear whether ADAMTS-4 was transcriptionally up-regulated in an inflammatory state. We are the first to demonstrate that the synthesis of ADAMTS-4 mRNA is induced by A β treatment. The A β -induced elevation of ADAMTS-4 suggests that ECM degradation occurs in AD brain, similar to that in articular cartilage of arthritic diseases. Previous studies have demonstrated that in the brain of AD patients the activities of MMPs responsible for degradation of ECM are higher than those in controls [16], and that ECM degradation occurring in senile plaques is caused by enzymes derived from astrocytes [3]. Furthermore MMPs were demonstrated to be induced in A β -treated astrocytes [6], and in central nervous system (CNS) aggrecan is suggested to be produced by astrocytes [2]. Therefore astrocytes are likely to be the cells responsible for regulation of ECM remodeling in brain; a phenomenon that would extensively change the environment of neurons.

The significance of ECM degradation induced by A β treatment has not been ascertained clearly. There are several possible reasons why ADAMTS-4 expression may be up-regulated, as follows: (1) ADAMTS-4 degrades ECM to make immuno-responsive cells, microglia in brain, migrate and influx into the lesions in response to inflammatory chemotactic factors. (2) As with articular cartilage in arthritic diseases, inflammatory pathways appear to be continuously operational in the activated glia in AD brain. This sustained inflammatory stimulus may increase activity of ADAMTS-4 over normal levels. (3) It was previously reported that ECM components accumulated in AD brain [4,7,23]. ADAMTS-4 may be induced in response to this abnormal accumulation of ECM.

In any case the increment of activity of this enzyme accelerates degradation of the brain ECM. This could result in loss of scaffold for the essential localization and maintenance of a neuron and its synapse and lead to dysfunction

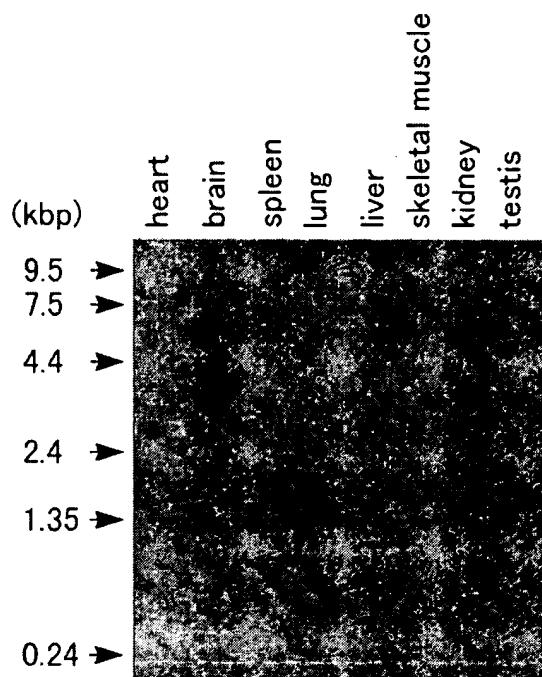


Fig. 3. Tissue blot analysis of rat ADAMTS-4 mRNA expression. Each lane represents 2 μ g of poly (A)+ RNA purified from heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis. Rat multiple tissue northern blot was hybridized with rat ADAMTS-4 cDNA probe and exposed for 72 h.

of intrinsic neural networks. ECM has also been suggested to play an important role in the regulation of cells mediated by release of bioactive fragments and growth factors, and adhesive signals [25]. ECM degradation in AD could create a deficiency of normal signals caused by these factors and lead to an exacerbation of the disease.

The current study demonstrates that ADAMTS-4 is transcriptionally induced by A β treatment and suggests that ECM degradation is promoted in AD brain. Consequently this may contribute to neuronal damage and development of chronic neurodegenerative disorders. Although additional studies are needed to distinguish the relative contributions of multiple neuroinflammatory phenomena occurring in AD brains and key factors in the progression of disease, ADAMTS-4 could be one critical factor in the exacerbation of neurodegeneration in AD. The regulation of the expression of ADAMTS-4 is likely to be important, as is the post-translational modification and maturation of this enzyme. A further understanding of ECM degrading enzymes including ADAMTS-4 and MMPs might lead to a therapeutic target in AD.

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Brief Screening Tests for the Diagnosis of Dementia: Comparison With the Mini-Mental State Exam

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Abstract: Dementia is a common and under-diagnosed problem among the elderly. An accurate screening test would greatly aid the ability of physicians to evaluate dementia and memory problems in clinical practice. We sought to determine whether simple and brief psychometric tests perform similarly to the Mini-Mental State Examination (MMSE) in screening for dementia. Using a retrospective analysis, a series of standard, brief, psychometric tests were compared with each other and to the MMSE as screening tests for very mild dementia, using DSM-III-R criterion as the gold standard. Two independent cohorts from the Baltimore Longitudinal Study of Aging and the Washington University Alzheimer's Disease Research Center were evaluated. We found that two brief and simple-to-administer tests appear to offer similar degrees of sensitivity and specificity to the MMSE. These are the recall of a five-item name and address, "John Brown 42 Market Street Chicago" and the one-minute verbal fluency for animals. Combining these two tests further improves sensitivity and specificity, surpassing the MMSE, to detect dementia in individuals with memory complaints.

Key Words: Blessed memory task, dementia screening, verbal fluency, MMSE

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Dementia is a common and often unrecognized problem in the elderly.¹ While estimated prevalence rates for dementia have shown some variability,² a prevalence of 20 to 30% in the eighth decade of life has been reported in several large studies.³⁻⁵ While pharmacologic treatment of dementia has been somewhat limited⁶ all patients should have an evaluation for reversible causes of dementia at the earliest possible stages, even though such reversible causes are not common.^{7,8} Moreover, the recognition of dementia in an early

stage would alert family members and caretakers to the need for possible intervention in areas such as driving,⁹⁻¹² household activities,^{13,14} and possible protection from financial predators. Since dementia has been shown to be a prime cause of long-term disability in the elderly,¹⁵ its early diagnosis allows both the patient and the family to make decisions regarding long-term health and financial matters at an earlier stage of the disease. Moreover, appropriate family interventions have also been shown to result in improved functionality, quality of life, and delayed nursing home placement in early-to mid-stage dementia.¹⁶ From the point of view of the primary care physician, a diagnosis of dementia should modify the approach to comorbid conditions and prescription practices in these patients.^{17,18}

While it may seem that a problem such as dementia should be self evident to the patient, family, and physician, multiple studies have shown that all 3 parties, especially the physician, can be unaware of the problem in 40 to 70% of cases, especially if it is mild to moderate.¹⁹⁻²⁴ Surprisingly, the degree of cognitive deficit that goes unrecognized often extends to subjects with Mini-Mental State Examination (MMSE) scores in the mid teens.^{25,26} Compounding the problem is the fact that patient complaints of memory loss in many cases are misleading^{25-27,29} because most truly demented patients are unaware of their deficits. Reports of family members are usually good indicators of cognitive difficulties, but are often absent in early to moderate stages of the disease.^{1,20,21,30}

Physician surveys have indicated that many primary care physicians believe that screening for dementia is an important goal³¹ but that time considerations prevent it.³²⁻³⁵ In addition, a brief and reliable approach to the evaluation of patient complaints of memory loss is also desirable.³⁶ Several screening tests have been designed to assess dementia. The most widely used is the Mini-Mental State Examination.^{37,38} However, in spite of its name and its value as a tool for following patients, the MMSE has not gained wide acceptance as a screening tool for "asymptomatic" patients or patients with suggestive signs of cognitive difficulties because it takes at least 5 minutes to perform, an unacceptably long duration for routine use in primary care practices, and requires paper and pencil to administer. Other tests that have been developed recently such as the "Seven Minute Dementia Screen"³⁹ improve on the sensitivity and specificity of the MMSE but take even longer to administer. The Memory Impairment Screen (MIS),⁴⁰ a test of free and cued recall, is somewhat shorter than the MMSE and has excellent published sensitivity and specificity in moderately demented patients. This test must be

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further examined in a group of mildly demented patients, those most likely to be living at home. However, it currently represents the best characterized and most promising screening tool.

We sought to evaluate and compare very brief psychometric tests as an alternative to the MMSE and MTS for widespread screening of dementia in older individuals and as a quick way to evaluate complaints of memory problems in this population. In this paper we demonstrate that two brief and simple-to-administer tests appear to offer similar degrees of sensitivity and specificity to the MMSE. These are the recall of a 5-item name and address, "John Brown 42 Market Street Chicago" (hereafter referred to as the Blessed Memory Test), and the 1-minute verbal fluency test for animals. The simplicity and accuracy of these tests may allow them to be used in a more widespread manner.

METHODS

Baltimore Longitudinal Study of Aging Cohort

The Baltimore Longitudinal Study of Aging (BLSA) is a prospective study of the effects of normal aging in community-dwelling volunteers and is conducted by the National Institute on Aging⁴¹ and is also supported by the Johns Hopkins Alzheimer's Disease Research Center. Studies of this cohort are conducted under the auspices of the Johns Hopkins IRB, which has approved all indicated procedures. Currently, 2476 subjects (1566 men, 910 women) have been enrolled in the BLSA since its inception in 1957. As of January 2003, a total of 229 incident cases of dementia have been identified among BLSA participants. For our initial comparison of multiple screening tests, 101 of the 229 demented subjects were excluded because of incomplete data. (In most cases this is due to the fact that they were early participants in the BLSA, before the current home evaluation was standardized.) An additional 31 were excluded due to the severity of their dementia MMSE <15 or Clinical Dementia Rating >1 (CDR)⁴² leaving 97 subjects for this study. Of the 97 demented subjects, 93 were white and 4 were African American; 57 were men and 40 were women. CDRs were not required on all subjects for inclusion in this study as it was a late addition to the BLSA testing protocol. However, in demented subjects on whom it was performed (approximately 60% of subjects) a CDR score >1 served as an exclusionary criterion; 54 of the 97 demented subjects are/were enrolled in the BLSA Autopsy program. The diagnosis of dementia was made at a consensus diagnostic conference using DSM-III-R criteria (1987).⁴³ At these conferences a full panel of neuropsychological tests and clinical data were available for review. No specific pattern of testing abnormality was required for diagnosis, although DSM-III-R criteria had to be met. Dementia was further classified by diagnostic category with use of National Institute of Neurologic and Communicative Disorders and Stroke—Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)⁴⁴ criteria for definite, probable, and possible Alzheimer disease (AD). An additional designation was used for participants who met clinical criteria for probable AD but did not complete neuroimaging or laboratory studies. These subjects were categorized as consistent with AD.

Demented subjects with evidence of significant cerebrovascular disease by history, examination, or imaging were designated as vascular or mixed vascular/AD dementia. Vascular dementia was defined using the State of California AD Diagnostic and Treatment Centers criteria.⁴⁵ All subjects designated as having vascular dementia met criteria for probable or possible vascular dementia. Of the 97 demented participants from the BLSA used for this analysis, 67% were diagnosed with probable, possible, or consistent with AD, 13% were mixed AD/vascular dementia, 8% were Parkinson's disease with dementia, and 12% were dementia unspecified.

To ensure that differences in the results of screening tests did not simply reflect the older age and lower educational status of demented subjects, we chose to match each demented subject by age and education to a randomly selected control subject, taken from the same cohort as the demented subjects, using frequency matching.⁴⁶ We divided the group into 7 age categories (each 5-year bins from 70–105) as well 5 education categories making a total of 35 bins from which subjects in one group were randomly matched to those in the other (control or demented). The matched control subjects were selected from the 417 fully evaluated controls (93% white, 63% male) over the age of 70 in the BLSA who had complete neuropsychological evaluations and were judged to be normal (CDRs were performed in 60% of this group and were required to be zero for inclusion in this analysis). Controls, like subjects, were not excluded if their CDR was not done. This control population is a 1:1 match rather than a 1:2 match because of the lack of 2 control subjects per demented subject in the older age range; 237 of the 417 control subjects are/were enrolled in the BLSA Autopsy program. For demented subjects with multiple test iterations over several years, we used the first series of tests following or coincident with the diagnosis of dementia to evaluate patients in their mildest state. For controls with multiple test iterations we used their last series of tests. An additional 107 participants in the BLSA had been labeled as having "mild cognitive impairment" at the time of their evaluation (mean MMSE 27.2 ± 2.3) because of either mild informant memory complaints in the absence of significant abnormalities on memory testing ($n = 58$) or because of deficits in 1 or more cognitive spheres by testing and complaint but no functional loss ($n = 49$).

Washington University Cohort

The Alzheimer's Disease Research Center (ADRC) at Washington University has been following a longitudinal cohort of mildly demented and non-demented control subjects since 1979. Studies of this cohort were conducted under the auspices of the Washington University IRB, which has approved all indicated procedures. The cohort includes data from 419 mildly demented and 159 control subjects, drawn from the community; 33% of the sample is male and 87% are white. Of the demented group, 348 had both a CDR less than or equal to 1 and a MMSE greater than or equal to 15 and were included in this analysis (all subjects in this cohort had CDRs). Approximately 70% of these subjects, described previously in Morris and Price,⁴⁷ Morris et al.,⁴⁸ Price and Morris,⁴⁹ and Berg et al.,⁵⁰ fulfill NINCDS-ADRDA criterion for "probable AD." The remaining 30% meet the criterion for "possible AD" on

one of two grounds: either they had another potentially dementing disorder (the two most frequent by far are depression and stroke) in addition to AD, or they had an atypical presentation (eg, prominent language disturbance). However, the primary dementing illness was determined clinically to be AD in these cases and the course of dementia was characteristic for AD.⁵¹ Diagnoses were determined by applying clinical diagnostic criterion for Dementia of the Alzheimer Type (DAT), as previously published.⁵² Subjects in this study had routine annual evaluations including a cognitive test battery that includes the 1-minute verbal fluency test for animals, the MMSE, and a modified Blessed. In contrast to the BLSA, the Washington University ADRC relied primarily on the CDR for clinical diagnosis, and used cognitive testing rather sparingly for diagnostic purposes. This cohort contains an excess of mildly demented participants compared with controls. Therefore we randomly matched 159 control subjects by age and education using frequency matching as described above to a group of 159 mildly demented subjects (from a total of 348 mildly demented subjects) (MMSE ≥ 15 , CDR ≤ 1), most of whom had probable AD.

Combined Cohorts

Given the similar results observed in the ADRC and BLSA cohorts and their overlapping testing and demographic variables, we combined the two cohorts for some analyses to allow greater statistical power and subgroup analysis; 378 of a total of 445 demented patients were matched by age and education to 378 controls using frequency matching. The matching was done across samples rather than just pooling matched samples. Sixty-seven demented patients were left out because of the lack of a suitable matched control. All mildly affected subjects and age-appropriate (>70) controls in both cohorts (445 demented; 613 controls) were included in a final unmatched analysis.

Tests and Analyses

At both sites, a standard version of the MMSE as described in Burns et al⁵³ was performed. In the BLSA cohort, the full Blessed Information–Memory–Concentration (BIMC) test was performed,⁵⁴ while the Washington University ADRC cohort underwent the Katzman Short Blessed.⁵⁵ Given the differences in these two forms of the Blessed, the delay between the encoding and recall of the Blessed Memory Test “John Brown 42 Market St Chicago” was about 2 minutes in the ADRC cohort and 4 minutes in the BLSA cohort. Participants were told that they were being asked to remember a person’s name and address. After hearing the name and address, the participants were asked to repeat the phrase, and were told that they will be asked to recall it later. For recall, the participants were asked to recall the name and address they had been given earlier. If no response was forthcoming, the patient was prompted with “John Brown ...?”. The use of a prompt did not change the scoring. One-minute verbal fluency for animals⁵⁶ was determined by asking the participants to name all the animals they could think of in 1 minute. Fish, birds, and insects were allowed. Scores for orientation to place and time (5 points each), 3-item recall (3 points), WORLD backward (5 points), and interlocking pentagons (1 point) were extracted

from the MMSE. The 2-item day and date score was a subset of the total MMSE time orientation score. Trails A and B were performed as described in Lezak.⁵⁷ Clockdrawing was scored as described in Burns et al.⁵³ Months of the year backward was taken from the Blessed and scored as 0, 1, or 2 depending on the number of errors made, and whether or not the participants self corrected. Cube drawing (copying) was scored as either correct or incorrect. Receiver operating characteristic (ROC) curves and 95% confidence intervals at different degrees of specificity were calculated for each test using MedCalc (Mariakerke, Belgium) software, version 7.0 (2002). MedCalc uses the principles for ROC curves described in Metz⁵⁸ and Zweig and Campbell.⁵⁹ ROC curves were compared by testing the statistical significance of the difference between the areas under the two ROC curves.⁶⁰ Linear regressions, slopes, and correlation coefficients were calculated using NCSS software (Kaysville, Utah).

RESULTS

Sample characteristics are presented in Table 1. As shown in Table 2 and Figure 1A, the three best and statistically identical tests for distinguishing mildly demented subjects from controls, using receiver operating characteristic (ROC) analysis in the BLSA sample, were the total score on the Mini-Mental State Examination, the 1-minute verbal fluency for animals, and the ability to remember the 5-item Blessed Memory Test (John Brown 42 Market St Chicago). Moreover, at high degrees of specificity (90 and 98%) the tests displayed similar sensitivities (approximately 70 and 50%, respectively), suggesting that 50% of mildly demented patients could be detected using extremely brief screening tests with few false positives. Combining the Blessed Memory Test with the 1-minute animal fluency increased the sensitivity to 79% with a 98% specificity. Comparison of demented and control populations without first matching to age and education modestly improved the sensitivity of all the diagnostic tests at each level of specificity (not shown). When we examined the Blessed Memory Task, the MMSE and the 1-minute verbal fluency for animals in subjects from the BLSA cohort excluded because of CDR >1 or MMSE <15 ($n = 31$; MMSE = 14.3 ± 2.7) we found all 3 tests performed well. Specifically, 3 or more errors on the Blessed Memory Task identified 29 of 31 subjects, a score of 23 or less on the MMSE identified 31 of 31 subjects, and a score of 8 animals or less in 1 minute identified 26 of 31 subjects.

To validate these findings, we examined another database of mildly demented subjects, obtained from the Washington University Alzheimer’s Disease Research Center (ADRC). As seen in Table 1, the mean MMSE and CDR of this population were similar to the BLSA population although the age and educational backgrounds were somewhat different. In the ADRC population, 100% of subjects had CDRs. Again as shown in Table 3 and Figure 1B, the results of brief psychometric testing on this cohort were almost identical to those obtained from the BLSA cohort, with the MMSE, 1-minute verbal fluency for animals, and Blessed Memory Test showing similar sensitivities and specificities.

Tests that did not manifest the same sensitivity and specificity as the MMSE either by ROC analysis or by sensitivity

TABLE 1. Demographic Characteristics of the ADRC and BLSA Cohorts

BLSA Matched Cohort							
Demented (n = 97)				Control (n = 97)			
Age	Education	MMSE	CDR (n = 58)	Age	Education	MMSE	CDR (n = 52)
84.2 ± 6.4	16.6 ± 3.3	23.9 ± 3.1	0.71 ± 0.24	83.8 ± 7.0	17.0 ± 2.9	28.6 ± 1.4	0
ADRC Matched Cohort							
Demented (n = 159)				Control (n = 159)			
Age	Education	MMSE	CDR	Age	Education	MMSE	CDR
78.5 ± 7.1	14.3 ± 3.3	23.9 ± 3.5	0.72 ± 0.25	78.0 ± 8.1	14.5 ± 3.2	28.7 ± 1.6	0
BLSA plus ADRC Matched Cohort							
Demented (n = 378)				Control (n = 378)			
Age	Education	MMSE	CDR	Age	Education	MMSE	CDR
79.8 ± 7.4	14.8 ± 3.0	23.8 ± 3.6	0.72 ± 0.25	79.5 ± 7.3	15.0 ± 2.9	28.5 ± 1.6	0
BLSA plus ADRC Unmatched Cohort							
Demented (n = 445)				Control (n = 613)			
Age	Education	MMSE	CDR	Age	Education	MMSE	CDR
80.0 ± 7.3	13.7 ± 3.7	23.5 ± 3.6	0.73 ± 0.24	78.6 ± 7.0	16.0 ± 3.0	28.6 ± 1.5	0
BLSA plus ADRC Matched Cohort, Age 80 or Above							
Demented (n = 188)				Control (n = 183)			
Age	Education	MMSE	CDR	Age	Education	MMSE	CDR
85.9 ± 4.6	15.3 ± 3.0	23.4 ± 3.6	0.76 ± 0.25	85.8 ± 4.2	15.6 ± 3.1	28.3 ± 1.6	0

All data are presented as the mean ± S.D. In the BLSA cohort, the number of subjects in each category who had a CDR is also indicated. Matched samples signify that demented and control subjects were matched 1:1 with regard to age and education.

at high specificity included the 3-item recall, clock drawing, and tests of orientation including day and date. All have been reported to be reasonable screening tools (See Discussion).

Since the results for the two cohorts and their demographic characteristics were quite similar we combined them to allow more statistical power and subgroup analysis. To avoid bias in combining the cohorts we analyzed them in both a matched and unmatched fashion. As shown in Table 4 and Figures 1C and 1D when the participants in the two cohorts were combined, and the demented and control subjects compared in either a matched or unmatched fashion, the MMSE, 1-minute verbal fluency for animals, and Blessed Memory Test again show similar ROC curves. The Blessed Memory Test showed a higher sensitivity than the other tasks at very high levels of specificity. Combining the verbal fluency and Blessed Memory Test (either one being positive constituting a positive screen) increased the sensitivity to over 70% with a specificity of 98%. Subgroup analysis (Table 4) showed that the advantages of the Blessed Memory Test, especially when combined with verbal fluency for animals, was also evident in the group whose age was 80 or above, those in greatest need of screening.

To present the data from the BLSA cohort in a more "real world" context we examined the utility of the 3 tests in the setting of subjective memory complaints. As part of their yearly CDR evaluation starting in 1996, BLSA subjects were asked whether they thought there had been a significant change in their memory since their prior evaluation. We evaluated the results of testing in 143 subjects over the age of

70 who responded affirmatively to the question of memory deterioration and were not previously known to be demented. Concurrent neuropsychological evaluation revealed that 47 of these 143 subjects were mildly demented at the time of their complaint. The MMSE identified 21 (45%) of these subjects with 1 false positive using a cutoff score of 23, the Blessed Memory Test identified 29 (62%) with no false positives using 3 errors as the cutoff value, and the 1-minute verbal fluency for animals identified 26 (55%) with 2 false positives. The combination of 3 errors on the Blessed Memory Test and 8 animals or less on the 1-minute verbal fluency identified 39 (83%; $P < 0.01$ compared with MMSE) of the demented subjects with only 2 false positives.

In establishing a screening test for dementia one of the most important attributes would be that it be insensitive in control subjects to the effects of age and education, the two items that most influence cognitive testing.^{57,61} In that regard both the MMSE and the verbal fluency for animals show a modest but statistically significant effect of age (MMSE slope = -0.06, $R^2 = 0.08$; Verbal Fluency slope = -0.2, $R^2 = 0.09$) and education (MMSE slope = 0.1, $R^2 = 0.034$; Verbal Fluency slope = 0.22, $R^2 = 0.024$) on test performance in non-demented controls ($N = 613$). In contrast, results from the Blessed Memory Test showed no effect of age (range 65–98) or education (range 8–24 yrs) on the results from control subjects. Moreover, the area under the ROC curve for the 3 tests as screening tools for dementia as well as their sensitivities at 90 and 98% specificity showed no sex differences in the matched or unmatched pooled samples used in Table 4.

TABLE 2. BLSA Sample (97 Matched Subjects)

	AUC \pm SE	95% CI	Sensitivity at 90% Specificity (95% CI)	Sensitivity at 98–99% Specificity (95% CI)
MMSE	0.89 \pm 0.02	0.84–0.93	72% (63–81) (≤ 26)	53% (44–63) (≤ 23)
John Brown err (Blessed)	0.88 \pm 0.02	0.83–0.92	66% (57–75) (≥ 2)	54% (45–65) (≥ 3)
Verbal fluency (Animals)	0.88 \pm 0.02	0.83–0.92	72% (63–81) (≤ 11)	50% (40–60) (≤ 8)
Combined (JBE + Animals)			(87%: 88% Spec) (2, 11)	79% (70–86) (3, 8)**
Trails B	0.84 \pm 0.03*	0.78–0.89	56% (48–67) (> 225 sec)	40% (32–50) (> 295 sec)
Recall of 3 items (MMSE)	0.81 \pm 0.03*	0.75–0.86	59% (50–68) (≥ 1)	37% (28–47) ($n = 0$)
Orientation (Time MMSE)	0.80 \pm 0.03*	0.74–0.85	67% (57–76) (≤ 4)	37% (27–47) (≤ 3)
Trails A	0.79 \pm 0.03*	0.73–0.85	53% (44–62) (> 73 sec)*	30% (21–39) (> 91 sec.)*
Day and date (MMSE)	0.76 \pm 0.03*	0.70–0.81	55% (45–65) (≤ 1)	25% (16–34) (0)*
Combined (JBE + Time)				61% (51–71)
Clock drawing (3:25)	0.77 \pm 0.03*	0.71–0.82	40% (30–50) (≤ 6)*	20% (12–29) (≤ 4)*
Verbal fluency S	0.72 \pm 0.03*	0.66–0.78	42% (32–53) (≤ 8)*	14% (7–22) (≤ 5)*
Orientation (Place MMSE)	0.70 \pm 0.03*	0.64–0.76	N.A.	24% (16–33) (≤ 3)*
Months backward	0.70 \pm 0.03*	0.64–0.77	40% (31–50) (≥ 1 Uncorrected Errors)*	
Cube drawing	0.64 \pm 0.04*	0.58–0.71	N.A.	N.A.
Interlocking pentagons	0.64 \pm 0.04*	0.57–0.70	N.A.	N.A.
World backward (MMSE)	0.64 \pm 0.04*	0.58–0.70	38% (27–46) (≤ 4)*	13% (7–21) (≤ 2)*

Area under the ROC curve (AUC) and 95% confidence intervals for that area for each of the indicated tests with regard to a diagnosis of dementia for 97 matched (age and education) control and demented subjects from the BLSA are indicated. Also shown is the sensitivity of each of the tests for the diagnosis of dementia at 90 and 98% specificity. N.A. implies that the test did not reach the indicated level of specificity. *Implies that differences between the indicated test and the MMSE is significant at the $P < 0.05$ level.

DISCUSSION

We have examined a series of simple psychometric tests for their ability to serve as screening tests for dementia in patients with mild disease. Given that patients with the mildest form of the disease are the ones most likely to be living at home autonomously and visiting their primary care physicians,^{20,62} screening tests should be targeted to that population. In that regard, our 2 data sets represent the mildest cohort of patients (judged either by MMSE and/or Blessed) to be examined with a range of screening tools. The sensitivity of both the Blessed Memory Test and the 1-minute verbal fluency for animals for detecting dementia in this group is approximately 70% at 90% specificity and 50% at 98% specificity. While these numbers do not represent an ideal test given the sensitivity values, these tests are at least as good as and much quicker than the MMSE, the most widely used test for screening for cognitive impairment. In addition, unlike the MMSE, the Blessed Memory Test and the 1-minute verbal fluency for animals do not require any specific form to administer. Given the almost complete lack of screening for dementia on the part of primary care physicians,^{33,34} our results show that this task can be accomplished quickly with a significant yield of patients who would often go undetected. Given the prevalence of this problem in patients in their 80s, this would represent a significant improvement over current practice. It is our contention that dementia screening tests for asymptomatic patients should emphasize ease and high specificity at the outset (which is why we chose to examine the sensitivity of these tests at 98% specificity) to encourage their use. In that regard, using tests at the highest level of specificity (3 or more errors on the Blessed Memory Test or 8 or less animals on the verbal fluency test) would be recommended. Given a prevalence of 20%, a positive screen at a 98%

specificity and 50% sensitivity has a positive predictive value of 80%, while at a specificity of 90% and a sensitivity of 70% a positive test has a positive predictive value of only 56%. In either case further evaluation would then be to confirm the diagnosis of dementia and determine its cause.

If increased sensitivity is desired, combining the Blessed Memory Test with the 1-minute verbal fluency for animals is a good approach, as it adds only 1 minute to the evaluation and substantially increases the sensitivity. In addition, the verbal fluency for animals task can serve as a useful distraction when administered between a patient's immediate recall of the 5-item Blessed Memory Test, and delayed recall of those items (see below).

In subjects with a higher pretest probability of dementia such as those with concerned family members or erratic behavior, a more sensitive but less specific cutoff such as 2 mistakes on the Blessed Memory Test or 11 or less animals in 1 minute (both with 90% specificity) might be more appropriate. Of note, our cutoff of 8 animals in 1 minute for screening purposes (98% specificity) differs significantly from what is considered "abnormal" (> 12) in the Boston Diagnostic Aphasia Battery.⁶³ Indeed less than 12 in our database (Table 1), results in a specificity of 90% with 70% sensitivity. It is only when specificity of 98% or better is sought that the value of 8 emerges.

We examined the characteristics of demented subjects who would go undetected using the combination of the Blessed Memory Task and the 1-minute verbal fluency for animals at 98% specificity (3 or more Blessed errors, 8 animals or less). The mean MMSE of this group was 27.1 ± 2.6 and the mean CDR was 0.58 ± 0.13 . Identifying this group would represent a daunting task for any screening procedure. A

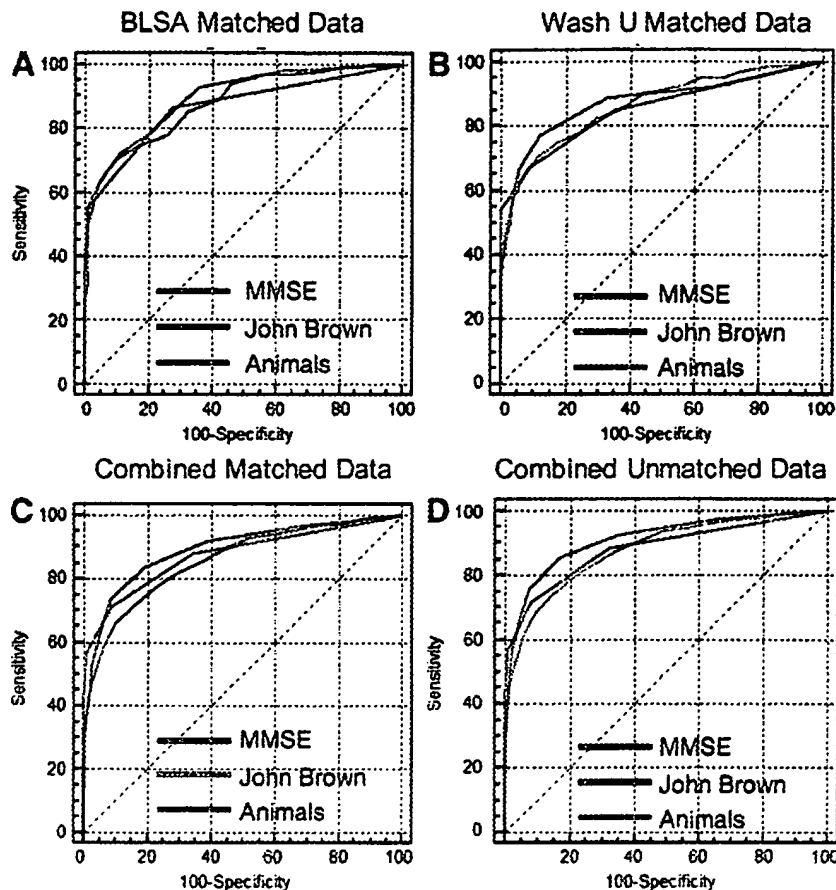


FIGURE 1. Sensitivity and specificity for the diagnosis of dementia of the MMSE, 1-minute verbal fluency for animals, and 5-item recall "John Brown 42 Market St Chicago" are presented in the form of ROC curves. A is the BLSA cohort, B the Washington University ADRC cohort, and C is the two cohorts combined. In A to C, demented and control subjects were matched for age and education. In D, the two cohorts are combined without regard to age and education. None of the differences in the curves are statistically significant.

similar problem was seen in evaluating subjects in the BLSA labeled as mild cognitive impairment (MCI; $n = 107$), a category of patients with lesser cognitive problems and no functional impairment. This group had a mean MMSE of 27.2 ± 2.3 . Alternative strategies will be needed to identify this group. However, given their lack of functional impairment and the absence of disease-modifying therapy, the need to identify these subjects is less acute. Practice-based studies indicate the prevalence of undiagnosed frank dementia in primary care is large and is a reasonable place to start.

In addition to the MMSE, two other tests have been proposed as screening tests for dementia. The first, called the MiniCog,⁶⁴ uses a combination of clock drawing and 3-item recall. Given that both performed at a significantly lower level than the Blessed Memory Test and animal fluency, it would be hard to envision their combination to be superior. A second screening test with published sensitivity and specificity is the Memory Impairment Screen (MIS).⁴⁰ Directly comparing our results with those published for the MIS is not possible at this point since our demented population was far less impaired (Mean Blessed of 14.7 (MIS) versus 8.8 (current study)) and our analysis included a greater number of examined subjects. A direct comparison of the two tests in a prospective study should be considered.

The value of the two tests we have identified as potential screening tests for dementia stem from their intrinsic properties. The Blessed Memory Test is internally cued since the subjects are asked to recall a name and address, the same stimulus they are given when the phrase is initially encoded. This type of encoding has been shown to be especially useful for detecting memory loss in demented patients.^{65,66} Moreover, the value of category fluency in the diagnosis of dementia, perhaps because of its dependence, in part, on temporal lobe structures, is known to be superior to both letter fluency and naming in the diagnosis of dementia.^{67,68} Like other recent studies, we found that clock drawing⁶⁹ and 3-word recall⁷⁰ were not particularly good as screening tests for the mildest stages of dementia.

One limitation of the current study is its lack of prospective design. Designing such a study, comparing The Blessed Memory Test and verbal fluency to other screening tests should be considered. However, the lack of such a study should not dissuade primary care doctors from screening for or evaluating dementia using these brief tests. The results for both are robust and hold up in two independent and different databases. It should be pointed out that a positive screening test does not make a diagnosis of dementia and should be evaluated further with a validated test such as the 7-minute

TABLE 3. Washington University ADRC Sample (159 Matched Subjects)

	AUC \pm SE	95% CI	Sensitivity at 90% Specificity (95% CI)	Sensitivity at 98–99% Specificity (95% CI)
MMSE	0.88 \pm 0.02	0.84–0.91	73% (66–80) (\leq 26)	45% (37–52) (\leq 23)
John Brown errors	0.86 \pm 0.02	0.82–0.90	65% (57–72) (\geq 2)	55% (46–62) (\geq 3)
Verbal fluency (Animals)	0.87 \pm 0.02	0.83–0.90	67% (61–75) (\leq 11)	48% (40–54) (\leq 8)
Combined (JBE + Animals)			(85%; 86% Spec) (2, 11)	68% (60–74) (3, 8)
Recall of 3 items (MMSE)	0.81 \pm 0.02*	0.77–0.84	56% (48–63) (\geq 1)*	36% (29–44) (0)*
Orientation (Time MMSE)	0.78 \pm 0.03*	0.73–0.82	60% (53–68) (\leq 4)	42% (34–50) (\leq 3)*
Day and date (MMSE)	0.77 \pm 0.02*	0.72–0.81	62% (54–69) (\leq 1)	26% (20–32) (\leq 0)*
Combined (JBE + Time)				62% (55–71) (3, 3)

Area under the ROC curve (AUC) and 95% confidence intervals for that area for each of the indicated tests with regard to a diagnosis of dementia for 159 matched (age and education) control and demented subjects from the Washington University ADRC are indicated. Also shown is the sensitivity of each of the tests for the diagnosis of dementia at 90 and 98% specificity. *Implies that differences between the indicated test and the MMSE is significant at the $P < 0.05$ level.

dementia screen³⁹ or similar diagnostic schemes,³⁶ which assess the multiple spheres necessary to establish a diagnosis of dementia. Moreover, an inquiry to the appropriate family members^{71–73} can be used to bolster a diagnosis of dementia or the patient could be referred to a memory disorders clinic.

Another limitation of the current study relates to the education level of our participant sample. Our study sample was decidedly skewed toward Caucasian subjects with education levels ranging from high school to post doctoral work. Even though our data suggest that the Blessed Memory Test showed no correlation between performance and educational background, the test should be further

evaluated in a broader sample of patients with lower education levels and a broader cultural and ethnic background. In that regard Duff Canning et al⁶⁷ have recently published data on a cohort of control and mildly demented subjects with considerably less education than our cohort, showing, as we have, that verbal fluency for animals (but not letter fluency) is a good discriminator of demented versus control subjects. Other tests such as the Blessed were not examined by Duff Canning and colleagues. In an accompanying editorial, Cummings⁷⁴ suggested that the use of fluency for action words might be useful for the diagnosis of Frontotemporal Dementia.

TABLE 4. ROC Curves for Dementia Screening Tests

Total Matched Sample (378 Demented; 378 Controls)				
MMSE	0.89 \pm 0.01	0.87–0.91	73% (68–77) (\leq 26)	44% (39–49) (\leq 23)
John Brown errors	0.88 \pm 0.01	0.86–0.91	72% (67–76) (\geq 2)	57% (52–62) (\geq 3)*
Verbal fluency (Animals)	0.86 \pm 0.02	0.83–0.88	66% (61–71) (\leq 11)	44% (39–48) (\leq 8)
Combined (JBE + Animals)			N.A.	71% (66–75) (3, 8)*
Orientation (Time MMSE)	0.78 \pm 0.01*	0.76–0.81	65% (61–70) (\leq 4)	42% (37–47) (\leq 3)
Combined (JBE + Time)				63% (58–68) (3, 3)*
Recall of items (MMSE)	0.78 \pm 0.01*	0.75–0.80	57% (52–61)* (\geq 1)	N.A.
Total Unmatched Sample (445 Demented; 613 Controls)				
MMSE	0.91 \pm 0.01	0.89–0.93	76% (72–80) (\leq 26)	48% (44–52) (\leq 23)
John Brown errors	0.88 \pm 0.01*	0.86–0.90	72% (68–76) (\geq 2)	57% (53–62) (\geq 3)*
Verbal fluency (Animals)	0.88 \pm 0.01*	0.86–0.90	68% (63–72) (\leq 11)	46% (41–50) (\leq 8)
Combined (JBE + Animals)			N.A.	73% (68–76) (3, 8)*
Orientation time (MMSE)	0.80 \pm 0.01*	0.78–0.83	66% (62–70) (\leq 4)*	45% (40–50) (\leq 3)
Combined (JBE + Time)				66% (61–70) (3, 3)*
Recall of 3 items (MMSE)	0.80 \pm 0.01*	0.78–0.83	54% (50–58) (\geq 1)*	34% (30–38) (0)
Combined Matched Sample Age 80 or Above (188 Demented; 183 Controls)				
MMSE	0.90 \pm 0.01	0.86–0.92	75% (69–81) (\leq 26)	46% (40–54) (\leq 23)
John Brown errors	0.88 \pm 0.01	0.84–0.90	73% (66–79) (\geq 2)	62% (55–68) (\geq 3)*
Verbal fluency (Animals)	0.86 \pm 0.02	0.83–0.90	71% (65–78) (\leq 11)	50% (43–57) (\leq 8)
Combined (JBE + Animals)			87% (77–94) (2, 11)	76% (69–81) (3, 8)*
Orientation time (MMSE)	0.83 \pm .02*	0.80–0.87	73% (67–80) (\leq 4)	47% (40–54) (\leq 3)
Combined (JBE + Time)				69% (62–76) (3, 3)*

Results of the ROC curves for various dementia screening tests generated by combining the cohorts of the BLSA and ADRC in a matched or unmatched fashion are shown. Also shown is the sensitivity of each of the tests for the diagnosis of dementia at 90 and 98% specificity. *Implies that the indicated test is superior to the MMSE at the $P < 0.05$. N.A. implies that the test did not reach the indicated level of specificity.

An additional matter worth discussing is the role of the recall delay and method of distraction performed as part of the performance of the Blessed Memory Test and its effect on the time required to perform the test. The two different datasets used slight variations on the test. The BLSA subject population used a 4-minute delay between encoding and recall, while the ADRC cohort used a 2-minute delay between encoding and recall. Each cohort included different psychometric tests between the encoding and recall of the memory test. The results of the test in the two populations were identical, implying that the exact amount of time delay and the exact content of the distraction do not appear critical. For this test to be truly useful to the primary care physician, the distraction between encoding and recall would need to be quite flexible, and include testing of verbal fluency for animals, if an increased sensitivity is desired, as well as standard inquiries about specific medical conditions or the general health of the patient if the most efficient use of the test is desired. In spite of these flexibilities, a minimum delay between encoding and recall of 2 minutes should be observed. In actual practice the time between encoding and recall could be used for something other than the test, such as history taking, decreasing dramatically the actual time specifically devoted to the test. Moreover, our data from BLSA participants do not show any evidence for a priming effect in control patients with repeat administration (not shown), an observation similar to that published by Davous et al,⁷⁵ who examined the effect of retesting on components of the Blessed. In that regard the test should be appropriate for serial examination of subjects over time.

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EDITOR-IN-CHIEF DISCLOSURE

Neither Dr. John C. Morris nor his family owns stock or has equity interest (outside of mutual funds or other externally directed accounts) in any pharmaceutical or biotechnology company. In 2003–2004, Dr. Morris had participated or is currently participating in clinical trials of antidementia drugs sponsored by the following companies: Elan, Eli Lilly and Company, Merck, and Wyeth. In the past year, Dr. Morris has served as a consultant or has received speaking honoraria for the following companies: Amgen, BristolMyersSquibb, Celera, Codman-Johnson & Johnson, Elan, Forest Labs, Janssen, Neurochem, Novartis, and Shionogi-Glaxo-Smith-Kline.

VASCULAR DEMENTIA AND ALZHEIMER'S DISEASE: THE UNSOLVED PROBLEM OF CLINICAL AND NEUROPSYCHOLOGICAL DIFFERENTIAL DIAGNOSIS

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The definition of clinical criteria for differential diagnosis of Vascular Dementia (VaD) and Alzheimer's disease (AD) remains controversial. A large group of patients selected was affected by chronic cerebrovascular disease classified as VaD or not (VND), according to DSM IV criteria. Neuropsychological performances of VaD patients were compared with a group of patients affected by probable AD, matched for age, education, and severity of disease. The comparison of performances did not reach statistical significance in single neuropsychological

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tasks. The results suggest that neuropsychological examination might not clearly differentiate between VaD and AD patients: the similar pattern of cognitive impairment is probably indicative of several common pathogenetic mechanisms.

Keywords Alzheimer's disease, differential diagnosis, neuropsychology, vascular dementia, vascular risk-factors

INTRODUCTION

Vascular Dementia (VaD) is traditionally considered a clinical syndrome of acquired cognitive impairment resulting from cerebral damage due to cerebrovascular etiology related to different mechanisms (Roman et al., 2002). VaD is the second most common type of dementia and its clinic-pathological classification includes different subtypes (Roman et al., 2002).

Despite the considerable degree of accuracy in diagnosing Alzheimer's disease (AD), the clinical differentiation with VaD and mixed dementia remains a matter of controversial opinions and one of the most challenging diagnostic issues.

Some previous studies stated that the cognitive deficits in VaD are different from AD especially for the domains of memory (Villardita, 1993; Tierney et al., 2001), attention/executive functions (Starkstein et al., 1996), and language (Powell et al., 1998). Indeed, other prior studies have failed to identify significant differences in these cognitive domains between VaD and AD patients (Almkvist et al., 1993; Rao & Howard, 1998). A recent neuropsychological study (Traykov et al., 2002) comparing two groups of patients affected by VaD and AD concluded that there were differences especially related to the executive functions, the latter markedly impaired in VaD as compared to AD, whose language function and non-verbal memory resulted, on the opposite, as more significantly damaged. Another neuropsychological comparison of two matched groups of patients with AD and VaD reported that language function and non-verbal memory performances were selectively more impaired in AD and that VaD patients performed better on the Trail Making Test (B) suggesting that, at variance with previous studies, there was no poorer performance on indices of frontal executive functioning in VaD (Baillon et al., 2003). These contrasting results further support the conclusion reached in some previous studies that reliable neuropsychological differentiation between VaD and AD is not possible (Almkvist et al., 1993).

AD is the most frequent form of primary dementia in the elderly population and its pathogenesis is only partially understood.

It is now recognized clinical history of stroke et al., 1997; Snowden

On the other hand infarcts, and prior stroke in the onset of VaD (I

In order to investigate, if any, in VaD study was carried out, of patients affected by neuropsychological performance of matched patients affected

SUBJECTS AND METHODS

After institutional review following two groups of patients.

CV were recruited stroke admitted to the independently from evidence

Inclusion criteria

- previous clinical history
- focal neurological
- no history or evidence
- risk factors for cerebro
- brain imaging showed

A total of 74 subjects Demographic data of

Patients affected cause of the limitation pairment, sub-arachnoid and absence of neuroi

CV were classified not demented (VND); drome was made according the diagnosis of Vasc

al examination might not clearly
the similar pattern of cognitive
common pathogenetic mechanisms.

agnosis, neuropsychology, vas-

ed a clinical syndrome of
brain damage due to cere-
broms (Roman et al., 2002).
and its clinic-pathological
et al., 2002).

in diagnosing Alzheimer's
) and mixed dementia re-
sults of the most challenging

the deficits in VaD are dif-
ferent memory (Villardita, 1993;
Starkstein et al., 1996),
prior studies have failed to
determine similarities between VaD and
AD (Roman et al., 1998). A recent neuro-
psychological study comparing two groups of patients
found that there are differences especially
in verbal memory in VaD as
well as in verbal memory resulted,
other neuropsychological
tests in AD and VaD reported
that performances were selec-
tively performed better on the
same with previous studies,
that executive functioning
results further support the
reliable neuropsychological
tests (Almkvist et al., 1993).
Dementia in the elderly popu-
lation.

It is now recognized that subjects with cardiovascular risk factors and a clinical history of stroke have an increased risk of both VaD and AD (Hofman et al., 1997; Snowden et al., 1997; Breteler et al., 2000).

On the other hand, in cerebrovascular patients diabetes mellitus, silent infarcts, and prior stroke have been considered relevant clinical determinants in the onset of VaD (Desmond et al., 2000).

In order to investigate further the differential pattern of cognitive impairment, if any, in VaD and AD patients a clinical and neuropsychological study was carried out, which identified Vascular Dementia in a selected group of patients affected by chronic cerebrovascular disease and assessed their neuropsychological performance in comparison with that of a selected group of matched patients affected by Probable AD.

SUBJECTS AND METHODS

After institutional review committee approval and informed consent the following two groups of patients were included: cerebrovascular (CV) and AD patients.

CV were recruited from a larger group of patients with definite ischemic stroke admitted to the Neurology Department of the authors' Institution independently from evidence of cognitive deterioration.

Inclusion criteria of CV were the following:

- previous clinical history of ischemic stroke;
- focal neurological signs;
- no history or evidence of major depression;
- risk factors for cerebrovascular disease;
- brain imaging showing ischemic lesions (two or more areas of infarction).

A total of 74 subjects (47 men and 27 women) were included in the study. Demographic data of enrolled subjects are reported in Table 1.

Patients affected by severe aphasia were excluded from the study because of the limitations on their cognitive assessment; visual or hearing impairment, sub-arachnoid or intraparenchymal haemorrhage, cerebellar stroke, and absence of neuroimaging data were other criteria of exclusion.

CV were classified into vascular demented (VaD; $n = 37$) and vascular not demented (VND; $n = 37$) subgroups; the diagnosis for dementia syndrome was made according to diagnostic criteria of DSM-IV. The criteria for the diagnosis of Vascular Dementia (VaD) were those of the International

Table 1. Characteristics of selected patients

Demographic data and MMSE score	Patients			
	Cerebrovascular disease (<i>n</i> = 74)	Vascular not-demented (<i>n</i> = 37)	Vascular demented (<i>n</i> = 37)	Alzheimer disease (<i>n</i> = 17)
Mean age \pm SD	71 \pm 8	70 \pm 8	72 \pm 8	68 \pm 7
Years of education	7 \pm 4	8 \pm 4	6 \pm 4	9 \pm 5
Males, <i>n</i> (%)	47 (62.67%)	28 (75.67%)	19 (51.35)	9 (52.94%)
Females, <i>n</i> (%)	27 (37.33%)	9 (24.32)	18 (48.65)	8 (47.06%)
Mean MMSE \pm SD	21.19 \pm 5.73	24.46 \pm 4.44	17.92 \pm 5.99	17.23 \pm 5.24

Classification of Disease, Tenth Revision (ICD-10) (World Health Organization, 1992).

AD patients (*n* = 17) were selected according to the NINCDS-ADRDA diagnostic criteria for probable Alzheimer's Disease (Mc Khann et al., 1994).

AD patients were selected to match the VaD patients on severity of cognitive impairment as assessed by the MMSE.

Neuroimaging showed diffuse and symmetrical brain atrophy for all AD patients. Mean age and educational level of each group of enrolled patients were not statistically different.

For each patient the main vascular risk-factors were classified into anamnestic, laboratory, and clinical factors (see Table 2).

Ischemic stroke was classified according to its mechanism as probably embolic (e.g., atrial fibrillation, cardiac valve pathology, or other cardiac embolic source) or probably thrombotic.

Location of stroke (right, left or bilateral) and atrophy (focal or diffuse) were also taken into account.

All patients underwent a neuropsychological assessment in order to explore a wide range of cognitive functions (Caltagirone et al., 1979).

The neuropsychological tasks included tests of memory, abstract thinking, praxis, executive, and visuo-spatial functions. Verbal memory functions were evaluated in the short- and long-term components using Rey's 15 words memory test (immediate and delayed recall and recognition).

Visual memory was assessed by a test of immediate visual memory and the memory's reproduction after 3 min of Rey-Osterrieth's figure. Raven's Colored Progressive Matrices were used to assess abstract thinking and visuo-spatial intelligence. Working memory was studied using digit and spatial spans in the forward and backward modalities. Spatial span was evaluated using

Table 2. Distribution of anamnestic, laboratory, and clinical factors in Vascular Dementia and Alzheimer's Disease

Risk factors
Anamnestic: <i>n</i> (%)
Familiarity for vasculopathy
Arterial hypertension
Diabetes
Hypercholesterolemia
Cigarette smoking
Myocardial infarction
Atrial fibrillation
Prior TIA
Prior ischemic stroke
Laboratory: mean (SD)
Haematocrit
Thrombocytes count
Glucose
Cholesterol
Triglycerides
Clinical: <i>n</i> (%)
Focal signs
Pyramidal
Extrapyramidal
Cerebellar
Sensory deficits
Sphincter troubles
Gait impairment
Primitive signs

Corsi's blocks forward and backward, assessed by the reproduction of the copy of Rey-Osterrieth's figure, and bucco-facial praxis with the use of the temporal rules in control tapping, and verbal fluency. Visuo-spatial functions were

Statistics

Fisher exact one-tailed test; Yates corrected Chi-square (*df* = 1) for frequencies >

Vascular demented (<i>n</i> = 37)	Alzheimer disease (<i>n</i> = 17)
72 ± 8	68 ± 7
6 ± 4	9 ± 5
19 (51.35)	9 (52.94%)
18 (48.65)	8 (47.06%)
7.92 ± 5.99	17.23 ± 5.24

(World Health Organiza-

o the NINCDS-ADRDA
(Mc Khann et al., 1994).
patients on severity of

brain atrophy for all AD
roup of enrolled patients

were classified into an-
2).

mechanism as probably
iology, or other cardiac

rophy (focal or diffuse)

essment in order to ex-
ne et al., 1979).

memory, abstract think-
erbal memory functions
its using Rey's 15 words
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liate visual memory and
errieth's figure. Raven's
tract thinking and visuo-
ag digit and spatial spans
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Table 2. Distribution of anamnestic, laboratory and clinical risk-factors for cerebrovas-
cular disease in VND and VaD

Risk factors	VND (<i>n</i> = 37/74)	VaD (<i>n</i> = 37/74)	<i>p</i>
Anamnestic: <i>n</i> (%)			
Familiarity for vasculopathy	6 (16.2)	11 (29.7)	0.1216
Arterial hypertension	24 (64.9)	28 (75.7)	0.2528
Diabetes	10 (27.0)	11 (29.7)	0.7965
Hypercholesterolemia	16 (43.2)	16 (43.2)	1.0000
Cigarette smoking	10 (27.0)	5 (13.5)	0.1609
Myocardial infarction	6 (16.2)	3 (8.1)	0.2710
Atrial fibrillation	5 (13.5)	5 (13.5)	0.8173
Prior TIA	10 (27.0)	11 (29.7)	0.7954
Prior ischemic stroke	16 (43.2)	26 (70.3)	0.0256
Laboratory: mean (SD)			
Haematocrit	43.26 (0.00)	39.31 (4.04)	0.1616
Thrombocytes count	199.98 (37.87)	218.71 (93.24)	0.5219
Glucose	110.00 (19.39)	106.09 (38.51)	0.6992
Cholesterol	249.85 (59.32)	206.71 (34.51)	0.0046
Tryglicerides	126.00 (107.38)	150.78 (109.20)	0.5365
Clinical: <i>n</i> (%)			
Focal signs	21 (56.8)	30 (81.1)	0.0134
Pyramidal	11 (29.7)	18 (48.6)	0.0768
Extrapyramidal	5 (13.5)	16 (43.2)	0.0078
Cerebellar	8 (21.6)	7 (18.9)	0.8180
Sensory deficits	13 (35.1)	21 (56.8)	0.0470
Sphincterial troubles	7 (18.9)	10 (27.0)	0.3706
Gait impairment	11 (29.7)	13 (35.1)	0.5617
Primitive signs	16 (43.2)	18 (48.6)	0.5629

Corsi's blocks forward and backward tests. Constructional functions were assessed by the reproduction of a geometrical figure representing a cube and the copy of Rey-Osterrieth's complex figure test. Motor control, ideomotor, and bucco-facial praxis were based on gestures-to-verbal command and imitation. The temporal rules induction test (Villa et al., 1995), Stroop's test, verbal control tapping, and verbal fluency were used to assess executive functions. Visuo-spatial functions were assessed by simple and double barrage.

Statistics

Fisher exact one-tailed test was used for comparison of frequencies = 1-3, Yates corrected Chi-square for comparison of frequencies = 4-5 and χ^2 test (*df* = 1) for frequencies >5. One way Analysis of Variance (ANOVA) was

used for continuous data. Statistical significance was obtained with Bonferroni's correction.

Statistics were calculated using "Statistic for windows," release 4.0 of Statsoft.

RESULTS

Anamnestic, laboratory, and clinical vascular risk-factors were not statistically different for VaD and VND subgroups (see Table 2).

Neuroradiological data showed that there were no significant differences in location of focal vascular lesions between VaD and VND patients.

The neuropsychological performances obtained by VND, VaD, and AD groups (see Table 3) were analyzed by means of one-way ANOVA (significance at a level of $p < .0012$).

One-way ANOVA analysis between VND and VaD patients demonstrated significant statistical differences in all the neuropsychological tests administered with the exception of oral praxis. Vice versa one-way ANOVA analysis between AD and VaD patients (level of significance: $p < .0012$) did not reach significative differences in any task.

DISCUSSION

The results of the present study showed no clear-cut differences comparing the cognitive performances of the two groups of VaD and AD patients. The comparison of these results with those obtained by previous studies was hampered by the heterogeneity of the neuropsychological tests used in different studies. However, among previous studies reporting some significant differences, patients affected by VaD did better than AD patients on a task of recognition memory (Tierney et al., 2001), whereas a recent study reported a significant frontal dysfunction in VaD in comparison with AD (Traykov et al., 2002). The latter result has been recently questioned by other authors (Baillon et al., 2003).

The present results confirm previous neuropsychological studies (Almkvist et al., 1993; Rao & Howard, 1998) that already found no significant detectable differences between VaD and AD.

There are three possible interpretations of these results: (1) neuropsychology is not sufficiently adequate to discriminate between these two separate forms of dementia; (2) the similarity of the pattern of cognitive impairment is probably underlain by AD and VaD also sharing several common

Table 3. Neuropsychological performance (VaD) and AD

Neuropsychological performance
Mean of scores
MMSE
Visual memory
Digit span forward
Digit span backward
Spatial span forward
Spatial span backward
Word fluency: phonologic
Word fluency: semantic
Raven's Progressive Matr
Rey's 15 mots: short term
Rey's 15 mots: recall
Rey's 15 mots: recognitic
Temporal rules induction
Ideomotor praxis
Oral praxis
Cube copy
Copying drawings with le
Line cancellation: numbe
Line cancellation: executi
Double barrage: accuracy
Double barrage: execution
Rey's figure: copy
Rey's figure: memory ex
Go no-go
Stroop test: number of er

*Statistical significance

pathogenetic mechanism of the present data.

The present results show that cognitive factors did not result in different groups. Therefore, one can necessarily exert a criticism that subjects with stroke have an increased risk of dementia (Snowdon et al., 1997).

obtained with Bonferroni's

windows," release 4.0 of

factors were not statistically significant (Table 2).

no significant differences between VND patients.

by VND, VaD, and AD one-way ANOVA (significant differences were not found).

VaD patients demonstrated significant differences in neuropsychological tests administered by one-way ANOVA analysis (post-hoc analysis: $p < .0012$) did not

show significant differences comparing VaD and AD patients. The results of the present study were hampered by the use of different studies. Significant differences, particularly on a task of recognition memory, were reported by Traykov et al., (2002). Other authors (Baillon et al., 2000) found no significant differences.

Neuropsychological studies (Almkvist and Nilner, 1995) found no significant differences between these two groups.

The results: (1) neuropsychological differences between these two groups; (2) the pattern of cognitive impairment; (3) sharing several common

Table 3. Neuropsychological performances of Vascular not-demented (VND) Vascular Dementia (VaD) and Alzheimer Disease (AD) patients

Neuropsychological performance	VND	VaD	AD	<i>p</i>	<i>p</i>
Mean of scores	(<i>n</i> = 37)	(<i>n</i> = 37)	(<i>n</i> = 17)	VND vs. VaD	VaD vs. AD
MMSE	26.44	17.91	17.23	0.0000*	0.6474
Visual memory	17.08	11.70	14.06	0.0000*	0.1032
Digit span forward	5.44	4.40	4.41	0.0003*	0.9816
Digit span backward	3.20	1.86	2.25	0.0000*	0.2534
Spatial span forward	4.33	3.03	3.43	0.0000*	0.3155
Spatial span backward	3.75	2.28	2.62	0.0000*	0.5099
Word fluency: phonological	21.70	9.25	13.73	0.0000*	0.1145
Word fluency: semantic	13.52	9.82	9.78	0.0000*	0.9815
Raven's Progressive Matrices	19.27	12.05	15.86	0.0000*	0.0646
Rey's 15 mots: short term	26.78	17.24	16.26	0.0000*	0.6549
Rey's 15 mots: recall	4.13	2.21	0.73	0.0000*	0.0194
Rey's 15 mots: recognition	85.40	75.02	73.31	0.0000*	0.7515
Temporal rules induction	28.15	18.40	24.20	0.0000*	0.1036
Ideomotor praxis	18.92	17.38	16.59	0.0000*	0.2890
Oral praxis	19.03	18.23	18.43	0.0043	0.7739
Cube copy	2.73	1.58	2.18	0.0000*	0.0751
Copying drawings with landmarks	18.24	13.51	14.62	0.0000*	0.5606
Line cancellation: number	58.42	56.83	55.62	0.0000*	0.6771
Line cancellation: execution time	72.43	128.11	68.07	0.0003*	0.0761
Double barrage: accuracy	90.30	76.03	77.87	0.0000*	0.7755
Double barrage: execution time	132.05	170.81	177.57	0.0000*	0.8770
Rey's figure: copy	25.50	12.23	15.82	0.0000*	0.3029
Rey's figure: memory execution	9.32	3.88	2.25	0.0000*	0.1995
Go no-go	8.41	4.86	5.28	0.0000*	0.7643
Stroop test: number of errors	2.68	8.58	9.50	0.0000*	0.7842

*Statistical significance at a level of $p < .0012$.

pathogenetic mechanisms; (3) both previous hypotheses contribute to explain the present data.

The present results showed that anamnestic, laboratory, and clinical risk-factors did not result in statistical difference between VaD and VND subgroups. Therefore, other factors and their related pathogenetic mechanisms necessarily exert a critical role in the clinical onset of VaD. It is now recognized that subjects with cardiovascular risk factors and a clinical history of stroke have an increased risk of both VaD and AD (Hofman et al., 1997; Snowden et al., 1997; Breteler, 2000).

The role of specific vascular risk-factors such as hypertension in the pathogenesis of AD in later life has been recently outlined (Forette et al., 1998; Kivipelto et al., 2001). A very high systolic or very low diastolic blood pressure seems to increase the incidence of AD (Qiu et al., 2003).

Other pathogenetic factors potentially involved in the clinical manifestations of dementia could be the reduced cerebral synaptic reserve that has been already considered crucial in the appearance of dementia among patients with cerebrovascular disease (Loeb & Meyer, 1996).

The critically attained threshold of cerebral hypoperfusion has been suggested as an important factor in the pathogenesis of AD (de la Torre, 1999; 2002).

The clinical onset of dementia in the elderly has been also related to the presence of a pre-existing cognitive decline referred to AD pathology and the clinical appearance of dementia could be viewed as a synergistic effect of degenerative and vascular lesions of the brain (Zekry et al., 2002).

On the other hand, the coexistence of an underlying degenerative pathology in patients affected by VaD might be a critical factor in determining a clinical and neuropsychological profile of dementia overlapping that of AD.

There are some limitations in the current study due to the clinical setting where a neuropathological correlative study of the cases included in the study was not possible.

A previous clinico-neuropsychological follow-up study completed with a post-mortem neuropathological verification on a large group of aged subjects resulted in the observation of neuropathological markers of Alzheimer's type with characteristic vascular lesions in a significant subgroup of the followed-up subjects who progressively developed clinical signs of severe dementia.

These recognized vascular lesions were mainly of the lacunar type and located in the subcortical white matter and basal ganglia (Snowdon et al., 1997). The prevalence of these vascular lesions in the basal ganglia, thalamus, and deep white matter might be functionally critical for the relevant connections between the basal ganglia and the neocortex when the latter has already been damaged by Alzheimer's type pathology.

Several genetic risk factors for AD are shared with VaD (Wakutani et al., 2002). The presence of at least an $\epsilon 4$ allele of the Apolipoprotein E, a definite risk-factor for AD, interacts with cerebrovascular risk-factors to increase the likelihood of developing either of the two types of dementia (Haan et al., 1999).

An increased plasma homocysteine level resulted in a strong, independent risk factor for the development of dementia and AD (Seshadri et al., 2002).

Whether these well-could play an important VaD in aged people still

In conclusion, the probably underlain by genetic mechanisms.

It remains to be ve chological tasks might de distinction between patie

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with VaD (Wakutani et the Apolipoprotein E, a scular risk-factors to in- types of dementia (Haan

d in a strong, independ- D (Seshadri et al., 2002).

Whether these well-defined, so as other more controversial risk-factors, could play an important role among causative factors of AD as well as of VaD in aged people still remains an open question.

In conclusion, the similarity of the pattern of cognitive impairment is probably underlain by AD and VaD also sharing several common pathogenic mechanisms.

It remains to be verified whether more adequate and skilled neuropsychological tasks might detect more subtle differences to allow a definite clinical distinction between patients with AD and those with VaD.

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Use of a monoclonal antibody to measure the surface expression of thrombospondin following platelet activation

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The radiolabelled monoclonal antibody, 5G11, directed against native thrombospondin, has been used to assess the surface expression of secreted thrombospondin on human blood platelets. Emphasis has been placed on studying the role of fibrinogen in this process. Unstimulated platelets bound low amounts of 5G11 (about 2000 molecules/platelet). Binding increased 2-fold and 5–7-fold after stimulation of platelets with ADP or thrombin (or ionophore A23187) respectively. Unstimulated platelets from patients deficient in α -granule proteins (gray platelet syndrome) bound baseline levels of 5G11. However, binding was not increased after activation. Thrombospondin expression on thrombin-stimulated normal platelets was for a large part divalent-cation-dependent and was not affected by AP-2, a monoclonal antibody to GPIIb-IIIa complexes. However, binding of 5G11 was some 50% lower when platelets were stimulated in the presence of Fab fragments of a polyclonal rabbit antibody to fibrinogen. This suggested either a direct binding of thrombospondin to surface-bound fibrinogen or a steric inhibition due to a close proximity of the two proteins. The fact that binding of 5G11 was at the lower limit of the normal range to the stimulated platelets of an afibrinogenemic patient specifically lacking detectable fibrinogen favoured the latter explanation. Thus, a major fibrinogen-independent pathway for thrombospondin expression must exist.

Thrombospondin is a 450-kDa glycoprotein consisting of three identical disulfide-linked chains [1, 2]. The protein was originally described as a major constituent released from platelets during thrombin stimulation [3]. Thrombospondin is now known to be synthesized by a variety of cells including megakaryocytes, endothelial cells, smooth-muscle cells, fibroblasts, monocytes and macrophages (reviewed in [4]). Despite this synthesis, the plasma level of thrombospondin is low and the bulk of the protein available to circulating platelets appears to be that secreted from α -granules (reviewed in [5]). Thrombospondin is a multifunctional molecule, containing binding sites for heparin [1, 2], fibrinogen [6–8] and fibronectin [7] amongst other proteins and ligands [4]. It is incorporated into the fibrin clot and may influence the structure of the fibrin network [9].

Following its secretion, part of the platelet thrombospondin becomes associated with the surface of the cell [10–14]. Here, it is available to participate in the surface contact interactions of platelets. In particular, thrombospondin has been identified as the endogenous 'lectin' that is present on the surface of thrombin-stimulated platelets [15, 16]. *In vitro* studies with purified proteins show that thrombospondin forms a stable complex with fibrinogen [6–8], while immunocytochemical studies [14] suggest that both proteins colocalize on activated platelets in close proximity to GPIIb-IIIa complexes, the surface receptor for plasma fibrinogen [5]. Interestingly, a polyclonal antibody to thrombospondin,

which inhibited platelet aggregation by thrombin, caused a decrease in the affinity of fibrinogen binding to its receptor [17].

Thus, a role for fibrinogen in the binding and/or expression of thrombospondin on activated platelets has been postulated. However, other experimental data suggest that thrombospondin expression may be independent of fibrinogen. For example, Gartner et al. [15] found a normal expression of membrane-bound 'lectin' on thrombin-stimulated platelets of a patient with congenital afibrinogenemia, where both plasma and platelet levels of fibrinogen were severely decreased. For this and other reasons (see Discussion), we decided to investigate the mechanism responsible for the expression of thrombospondin on stimulated platelets and, in particular, the role of fibrinogen in this process. In so doing we have used a monoclonal antibody to thrombospondin to quantify its binding to platelets after secretion, and have compared results for normal platelets to those obtained using platelets from patients with selected α -granule deficiencies.

MATERIALS AND METHODS

Materials

Non-immune rabbit immunoglobulins and rabbit immunoglobulins to human fibrinogen were purchased from Dakopatts a/s (Glostrup, Denmark). The IgG were hydrolyzed with papain at a 1:100 (w/w) ratio in the presence of 2 mM EDTA and 10 mM cysteine. After 4 h at 37°C, 20 mM *N*-ethylmaleimide was added. The samples were extensively dialyzed against 0.01 M phosphate buffer pH 8.0 and

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Abbreviations. mAb, monoclonal antibody; GP, glycoprotein.

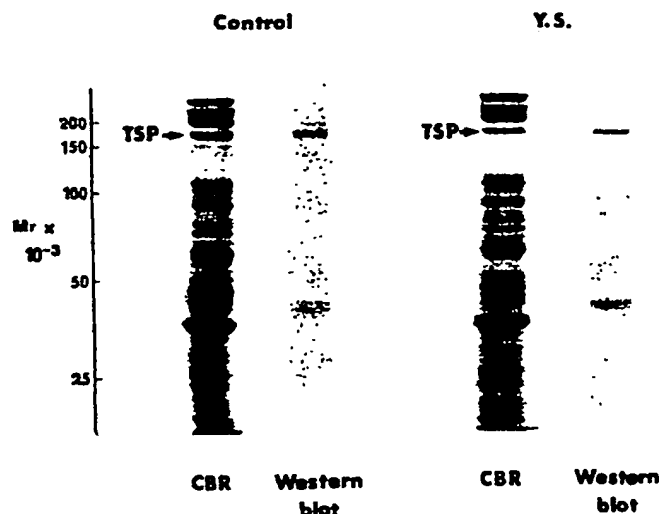


Fig. 1. *Specific binding of 5G11 to thrombospondin (TSP)*. Washed platelets from a control and an afibrinogenemic patient (Y. S.) were solubilized in 2% SDS in the presence of 5% (v/v) 2-mercaptoethanol. Samples (80 µg protein) were electrophoresed on a 5–12% gradient acrylamide slab gel. The proteins were either stained with Coomassie brilliant blue R250 (CBB) or electrophoretically transferred to nitrocellulose membrane and incubated with 1 ml (500 000 cpm) of ^{125}I -labelled 5G11. The labelled bands were visualized by autoradiography

passed through protein-A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) to remove Fc fragments and non-hydrolyzed IgG. The anti-fibrinogen Fab fragments were affinity-purified on a fibrinogen-Sepharose CL 4B column. (The details of the preparation will be given elsewhere; Legrand, C., Dubernard, V. and Nurden, A. T., unpublished results.) Their protein concentration was determined spectrophotometrically using an absorption coefficient $A_{280\text{nm}}^{1\%}$ of 1.53. More than 95% of the purified Fab fragments formed Fab:fibrinogen complexes precipitated with 9% poly(ethyleneglycol) 6000 (Fluka AG, Buchs, Netherlands). The murine anti-(GPIIb-IIIa) monoclonal antibody (AP-2) has been previously characterized [18]. Non-immune mouse and rat IgG were purchased from Nordic Immunology (Tilburg, Netherlands). ^{14}C -labelled 5-hydroxytryptamine creatinine sulphate (50–60 Ci/mol) and iodine-125 (13–17 mCi/µg) were from Amersham International (Amersham, UK). ADP, α -thrombin (3000 NIH units/mg protein) and hirudin (18000 units/mg protein) were from Sigma Chemicals Co. (St Louis, MO). γ -Thrombin was the generous gift of Drs M. C. Guillin and M. Jandrot-Perrus (Hôpital Bichat, Paris). The calcium ionophore A23187 was from Calbiochem Behring (La Jolla, CA) (a 2 mM stock solution was prepared in 70% ethanol). Reagents for SDS/polyacrylamide gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA, USA).

Production of monoclonal antibodies against human thrombospondin

Calcium-replete thrombospondin was purified from the supernatant of thrombin-activated human platelets essentially as described by Lawler and coworkers [19]. Monoclonal antibodies (Mabs) were produced in collaboration with Immunotech (Marseille, France) following the Köhler and Milstein procedure [20]. Full details will be presented elsewhere (Kieffer, N., Bourgois, A. and Nurden, A. T., unpublished results). In brief, LOU rats were immunized with 50 µg thrombospondin followed by a second boost of 25 µg injected

3 weeks later. Spleen cells from these animals were fused with the myeloma cell line IR983F. Positive hybridomas were detected by solid-phase radioimmunoassay using purified thrombospondin. The selected 5G11 clone was propagated by intraperitoneal injection into pristane-primed LOU rats. The mAb was purified from ascites fluid by precipitation with 40% saturated ammonium sulfate followed by gel filtration on DEAE-Trisacryl and elution with increasing concentrations of Tris/HCl, pH 8.0. The isolated IgG belonged to the IgG2a class [21]. Preliminary studies using the western blot procedure have located the 5G11 epitope on a 15-kDa fragment generated by trypsin digestion of purified thrombospondin in the presence of EDTA [21]. This fragment originates from the heparin binding domain of the molecule [22]. The use of 5G11 to detect membrane changes in acquired platelet disorders has already been reported [23].

Protein labelling

IgG were radiolabelled with ^{125}I by the chloramine T procedure [24] using carefully controlled conditions. Free ^{125}I was separated from the sample by gel filtration through a PD-10 column filled with Sephadex G-25 (Pharmacia). More than 98% of the radioactivity eluted with the antibody was precipitated by 20% trichloroacetic acid and a specific activity of approximately 500 cpm/ng protein was obtained. Protein concentration was calculated from absorbance at 280 nm and samples were stored at -80°C in the presence of 2 mg ml^{-1} bovine serum albumin.

Preparation of washed platelets

Blood (6 vol.) from medication-free human volunteers was taken into acid/citrate/dextrose (130 mM citric acid, 126 mM trisodium citrate, 110 mM dextrose) (1 vol.) and centrifuged for 15 min at $120 \times g$ and 20°C . Platelet-rich plasma was removed by aspiration and incubated for 30 min at 37°C with $0.6\text{ }\mu\text{M}$ 5-hydroxy[^{14}C]tryptamine in the presence of $25\text{ }\mu\text{g ml}^{-1}$ apyrase (grade I, Sigma). Platelets were isolated by re-

peated centrifugations in a modified Tyrode's buffer pH 6.5, containing 25 $\mu\text{g ml}^{-1}$ apyrase and 100 nM prostaglandin E1 [25]. The pellet was resuspended to a final platelet count of $2.5 \times 10^8 \text{ ml}^{-1}$ in a modified Tyrode's buffer, pH 7.4, containing 5 mM Hepes, 2 mM CaCl_2 , 1 mM MgCl_2 and 3.5 mg ml^{-1} bovine serum albumin (Tyrode/albumin). When the release of 5-hydroxy[^{14}C]tryptamine was studied, chlorimipramine (Ciba Geigy, Basel, Switzerland) was added to a final concentration of 3 μM . Secretion of β -thromboglobulin was measured by radioimmunoassay using a commercial kit (Amersham, UK).

Patients with deficiencies of secreted platelet proteins

Platelets from two patients with the gray platelet syndrome (H.B. and C.O.) and one patient with congenital afibrinogenemia (Y.S.) were investigated by courtesy of Professors J. Caen and C. Soria (Hôpital Lariboisière, Paris). In the gray platelet syndrome, α -granules and their contents are severely deficient whilst congenital afibrinogenemia is characterized by a specific fibrinogen deficiency [26]. The case history of patient H.B. was detailed by Levy-Toledano et al. [27]. The platelet β -thromboglobulin content was < 2% of that of normal platelets [27] while no release of fibrinogen or thrombospondin was detected upon thrombin stimulation [28]. Electron microscopy revealed a similar α -granule deficiency in platelets of patient C.O. [29], while biochemical studies confirmed a specific and severe deficiency of α -granule proteins (Nurden, A. T., unpublished findings). No fibrinogen was detected by an ELISA assay either in the plasma or in the platelets of patient Y.S. with congenital afibrinogenemia [30] while platelet β -thromboglobulin content (70 $\mu\text{g}/10^9$ platelets) was in the normal range ($76 \pm 16 \mu\text{g}/10^9$ platelets, $n = 8$).

Western blot procedure

Human platelets were solubilized with 2% SDS, the disulfides reduced in the presence of 5% 2-mercaptoethanol, and samples (80 μg protein) electrophoresed on 5–12% gradient acrylamide slab gels using the procedure previously described by us [26, 31]. Other details of the immunoblot procedure, including the electrophoretic transfer of proteins from unstained polyacrylamide gels to nitrocellulose membranes, were as reported by Kieffer et al. [32]. Individual nitrocellulose membrane strips were incubated for 2 h with 1 ml (500 000 cpm) of ^{125}I -labelled 5G11. The washed strips were dried and autoradiography performed as reported [32].

Binding assay for platelet-bound thrombospondin

Unstirred platelet samples ($2.5 \times 10^8 \text{ ml}^{-1}$) were incubated for 3 min at 37°C with different agonists as detailed in the figure and table legends. At the end of the incubation, hirudin was added in a tenfold excess (U/U) to those samples stimulated with α -thrombin. Aliquots were taken for the determination of 5-hydroxy[^{14}C]tryptamine or β -thromboglobulin release. The platelets were then diluted to $1.25 \times 10^8 \text{ ml}^{-1}$ and ^{125}I -labelled 5G11 was added as indicated (see Results). Incubation was for up to 30 min at room temperature. At selected times, triplicate 0.1-ml aliquots were removed and the platelets sedimented through 0.5 ml 20% (w/w) sucrose by centrifugation for 5 min in an Eppendorf centrifuge. The radioactivity associated with the pellets was counted in a gamma 7000 counter (Beckman Instruments Inc., Fullerton, CA). Controls were performed by centrifuging ^{125}I -5G11 in

Table 1. 5G11 binding to platelets

Platelets ($2.5 \times 10^8 \text{ ml}^{-1}$) in Tyrode/albumin were incubated with various agonists for 3 min at 37°C. With α -thrombin the reaction was stopped by the addition of hirudin in a tenfold excess (U/U). Samples were taken for measurement of 5-hydroxy[^{14}C]tryptamine or β -thromboglobulin release (see Materials and Methods). Platelets were then diluted to $1.25 \times 10^8 \text{ ml}^{-1}$ and incubated for 30 min at room temperature with 20 $\mu\text{g}/\text{ml}$ radiolabelled 5G11. Results are the mean \pm SD. n = the number of experiments where 5G11 binding and 5-hydroxy[^{14}C]tryptamine release were measured in parallel. β -Thromboglobulin release was determined in eight selected experiments. The mean platelet content of β -thromboglobulin was $76 \pm 16 \mu\text{g}/10^9$ platelets

Agonist	5G11 binding	5-Hydroxy[^{14}C]-tryptamine release	β -Thromboglobulin release
	molecules/platelet	%	
0 ($n = 54$)	1900 ± 1100	3.3 ± 1.1	7.9 ± 3.4
ADP 10 μM ($n = 6$)	3300 ± 700	5.4 ± 1.2	15.5 ± 2.8
α -Thrombin 0.5 nM ($n = 11$)	9400 ± 3000	42.3 ± 16.7	50.6 ± 12.8
1 nM ($n = 34$)	14100 ± 7500	62.0 ± 15.9	64.8 ± 10.6
γ -Thrombin 25 nM ($n = 34$)	12600 ± 5100	61.1 ± 12.2	64.3 ± 7.3
A23187 0.25 μM ($n = 34$)	10700 ± 4800	54.1 ± 11.2	70.8 ± 8.4

the absence of platelets. The background radioactivity was subtracted from the total radioactivity in the platelet pellet.

RESULTS

Specificity of mAb 5G11

SDS-solubilized platelet proteins separated by SDS/polyacrylamide gel electrophoresis were transferred to nitrocellulose membrane and incubated with ^{125}I -labelled 5G11 in a western blot assay. In the absence of disulfide reduction 5G11 bound to a band that barely penetrated the 5% separating gel. After disulfide reduction, 5G11 recognized a polypeptide of 170 kDa corresponding to the reduced chain of thrombospondin [28] (Fig. 1). In addition, a band of 40 kDa was faintly labelled. This band was also observed with radiolabelled non-immune rat IgG. It may correspond to a platelet Fc receptor for IgG [33]. Platelets from the afibrinogenemic patient (Y.S.) gave a band in the normal position for thrombospondin. However, quantitative densitometric scanning of Coomassie-blue-stained gels revealed that the thrombospondin content of Y.S. platelets was about 70% that of the corresponding control platelet sample. Platelets from the two gray platelet syndrome patients (H.B. and C.O.) contained no detectable thrombospondin (not illustrated), thus confirming previous studies [28].

Binding of mAb 5G11 to normal human platelets

The binding of 5G11 to washed platelets before and after stimulation was first characterized. Platelets were at

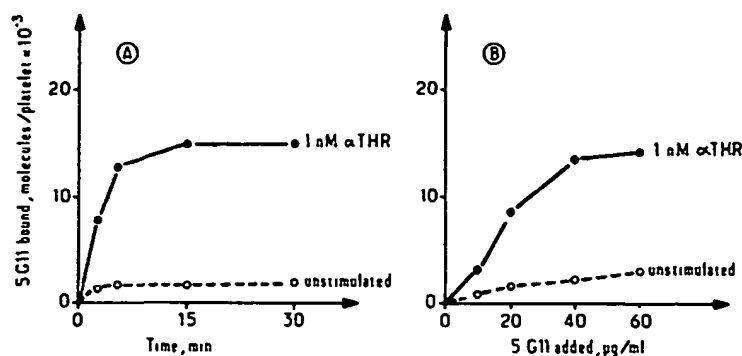


Fig. 2. Binding of 5G11 to unstimulated washed platelets. Platelet suspensions ($2.5 \times 10^8 \text{ ml}^{-1}$) were incubated at 37°C with or without 1 nM α -thrombin (α -THR). After 3 min hirudin was added in a tenfold excess (U/U) and the platelets diluted to $1.25 \times 10^8 \text{ ml}^{-1}$ with Tyrode/albumin. (A) 20 $\mu\text{g/ml}$ ^{125}I -5G11 was added and the samples incubated at room temperature for different times. (B) Increasing concentrations of ^{125}I -5G11 were added and the incubation was for 30 min. Platelets were then sedimented and the associated radioactivity measured as described in Materials and Methods

$2.5 \times 10^8 \text{ ml}^{-1}$, i.e. their concentration in circulating blood. A 3-min stimulation corresponding to the time required for maximal aggregation of platelets in the aggregometer cuvette was chosen. The platelets were then incubated at $1.25 \times 10^8 \text{ ml}^{-1}$ for 30 min at room temperature with 20 $\mu\text{g/ml}$ 5G11 i.e. its K_d value (see below). A low binding, corresponding to 1900 ± 1100 molecules per platelet was measured on unstimulated cells (Table 1). Under identical conditions 880 ± 710 molecules of non-immune rat IgG/platelet were sedimented. The binding of 5G11 was dramatically increased upon thrombin stimulation (Fig. 2). No increase in the sedimentation of non-immune IgG was observed (not shown). Binding of 5G11 to thrombin-stimulated platelets was rapid and approached equilibrium after 5 min (Fig. 2A). For platelets stimulated with 1 nM α -thrombin, binding was saturated at a concentration of 40 $\mu\text{g/ml}$ antibody (Fig. 2B). Table 1 shows that the binding of 5G11 to stimulated platelets was related to the ability of the agonist to induce the release reaction. ADP was poorly effective while platelet stimulation with α -thrombin, γ -thrombin and the calcium ionophore A23187 all resulted in a 5–7-fold increase in the binding of 5G11 to platelets. In each case up to 70% of dense body and α -granule release had occurred. Phase-contrast microscopy confirmed the absence of platelet aggregates in the unstirred platelet suspensions. As a result of these studies, a dose of 1 nM α -thrombin was chosen for most experiments.

Binding of 5G11 to patients' platelets

Platelets from the patients with the gray platelet syndrome (H. B. and C. O.) and congenital afibrinogenemia (Y. S.) were isolated and stimulated with 1 nM and 5 nM α -thrombin. Here, a high dose of α -thrombin was also used to overcome a previously reported decreased sensitivity of the release reaction from these patients' platelets [27]. The results are presented in Table 2. The platelets from the two patients with the gray platelet syndrome bound baseline levels of 5G11 but no increase was observed after platelet stimulation. A decreased thrombin-induced release of 5-hydroxy[^{14}C]tryptamine from the platelets was confirmed. The platelets from the afibrinogenemic patient underwent a normal release reaction. On two different occasions, at a three-month interval, the binding of 5G11 to his thrombin-stimulated platelets was at the lower end of the normal range. This may be related to the slightly lower thrombospondin content of the platelets of this patient (see Fig. 1).

Inhibition of binding of 5G11 to normal platelets

The binding of 5G11 to thrombin-stimulated normal platelets was decreased by more than 75% ($p < 0.01$) when platelet activation was performed in the presence of EDTA (Fig. 3). In these experiments the release reaction was not affected by divalent cation chelation (values in parentheses). The binding of 5G11 to platelets stimulated in the presence of the synthetic peptide Gly-Pro-Arg-Pro, which prevents the formation of fibrin polymers [8, 35], was not modified when platelets were stimulated with 1 nM α -thrombin and only slightly decreased when the platelets were stimulated with 5 nM α -thrombin. The concentration of Gly-Pro-Arg-Pro (a 1000-fold greater molar concentration over that of platelet fibrinogen, calculated on the basis of approximately 88 μg fibrinogen/ 10^9 platelets [34]) was in large excess over that required to inhibit fibrin polymerization completely [8, 35]. Gly-Pro-Arg-Pro did not modify the binding of 5G11 to platelets stimulated with 25 nM γ -thrombin, a hydrolytic product of α -thrombin that is unable to induce fibrinogen-to-fibrin transformation.

Platelets were also activated in the presence of an excess of affinity-purified anti-fibrinogen Fab fragments. In a previous study these have been shown to inhibit ADP-induced platelet aggregation completely when added in a 20-fold molar excess over the concentration of fibrinogen in the suspension and to bind maximally to thrombin-stimulated platelets within 5 min of incubation [36]. In the present experiments the anti-fibrinogen Fab were used at a concentration of 0.1 mg/ml, i.e. an approximate 40-fold molar excess over the calculated platelet fibrinogen content. Under these conditions they decreased by 50% the binding of 5G11 to platelets stimulated with 1 nM α -thrombin (Fig. 4). No inhibition was observed with non-immune Fab fragments. The specificity of this action was confirmed by their lack of effect on thrombospondin expression by platelets of the patient with congenital afibrinogenemia (Fig. 4). As judged by the release of 5-hydroxy[^{14}C]tryptamine, the antifibrinogen Fab fragments did not interfere with the stimulation of the platelets. No further inhibition was noted on increasing either the concentration of the anti-fibrinogen Fab fragments or the time of incubation of these fragments with the platelets. An identical inhibition was obtained when platelets were stimulated with 25 nM γ -thrombin (not shown).

Finally Fig. 4 shows that no inhibition of thrombospondin expression was observed when platelets were stimulated in the

Table 2. 5G11 binding to platelets of patients with deficiencies of α -granule proteins

Platelets ($2.5 \times 10^8 \text{ ml}^{-1}$) were processed as described in the legend to Table 1. Patients H. B. and C. O. were examined once. Y. S. was examined on two occasions and similar results were obtained. Results from one experiment are given. The control values are those obtained for the normal donors studied in parallel ($n = 8$)

Thrombin concentration	Platelet samples	5-Hydroxy[^{14}C]tryptamine release	β -Thromboglobulin release	5G11 binding
nM		%		molecules/platelet
0	control	3.3 ± 1.5	2.4 ± 1.7	2000 ± 1600
	H. B.	2.8	n.d.	2300
	C. O.	1.0	n.d.	2600
	Y. S.	1.4	0	1600
1	control	63.0 ± 14.5	62.3 ± 16.7	16700 ± 6100
	H. B.	30	n.d.	1500
	C. O.	20.8	n.d.	2200
	Y. S.	67.7	66.4	9100
5	control	74.0 ± 8.5	67.4 ± 10.8	14200 ± 4300
	H. B.	47	n.d.	2400
	C. O.	43	n.d.	1900
	Y. S.	77.5	70.3	9200

presence of AP-2, a murine monoclonal antibody to GPIIb-IIIa complexes used here at a saturating concentration [18]. Under these conditions AP-2 inhibits by more than 85% the binding of plasma fibrinogen to thrombin-stimulated platelets but does not interfere with the surface exposure of intracellular fibrinogen (Legrand, C., unpublished results).

DISCUSSION

Thrombospondin is secreted from platelet α -granules from where it binds to surface receptors and plays a role in platelet aggregation (see introduction). In support of a possible functional role, platelet aggregation is inhibited by monoclonal and polyclonal antibodies to thrombospondin [17, 37].

Until recently the presence of thrombospondin on the surface of platelets has been assessed by surface labelling [10, 11] or by immunofluorescence [12] and immunocytochemical techniques [13, 14]. However, this does not allow a quantitative estimation of the protein on the surface of the cell. In a recent study Aiken et al. [38] using TSP1-1, a mouse monoclonal antibody to purified thrombospondin, and different platelet concentrations, have estimated that from 7000 to 60000 molecules of the antibody bound per platelet after thrombin stimulation. Here, we have measured the binding of 5G11, a rat monoclonal antibody, to platelets activated by various stimuli. The specificity of this antibody for thrombospondin was established by studying its interaction with platelet proteins using an immunoblot procedure.

A binding assay was developed with platelets at a concentration of $2.5 \times 10^8 \text{ ml}^{-1}$, i.e. their approximate concentration in circulating blood, and radiolabelled 5G11 at $20 \mu\text{g/ml}$ ($0.13 \mu\text{M}$), i.e. a value similar to its apparent K_d (see Fig. 2). As shown by others [23], we found that low amounts of the antibody, of the order of 2000 molecules/platelet, bound to unstimulated cells. This was significantly in excess of that observed with a non-immune rat IgG which indicated that surface-bound thrombospondin was being detected. This binding could reflect either surface exposure of low amounts of endogenous thrombospondin during platelet isolation or thrombospondin normally present on circulating platelets which may come from the low amounts that occur in plasma

[39]. The fact that platelets isolated from two patients with the gray platelet syndrome bound baseline levels of 5G11 similar to normal platelets favours the second hypothesis. Although thrombospondin can not be detected in the platelets of these patients [26, 28], it is present in normal or even increased amounts in their plasma (Kieffer, N., unpublished results).

The binding of 5G11 to normal platelets was increased upon platelet stimulation providing that the release reaction had occurred. A 5–7-fold increase was observed when platelets were stimulated with α or γ -thrombin or with the calcium ionophore A23187. This increase was not observed with the platelets of the two patients with the gray platelet syndrome. The value found in our study for thrombin-stimulated platelets incubated with a half-saturating concentration of the antibody (14100 ± 7500 molecules 5G11 per platelet, $n = 34$) is similar to the values published by George et al. [23] and Aiken et al. [38]. Since thrombospondin is probably composed of three identical polypeptide chains [1, 2] and 5G11 reacts with the reduced chain in immunoblotting, up to three IgG molecules may bind to each molecule, and even more if the antibody recognizes a repeating unit [2]. This will depend on the accessibility of the binding sites. Thus, the number of thrombospondin molecules expressed on the surface of activated cells may be less than the absolute value determined using the monoclonal antibody.

In agreement with previously published data [10, 13, 38] we found that divalent cations were required for a full expression of endogenous thrombospondin on the surface of thrombin-activated platelets. This is also a requirement for the binding of fibrinogen, fibronectin or von Willebrand factor to GPIIb-IIIa complexes [5]. Although purified thrombospondin has been shown not to interact directly with purified GPIIb-IIIa molecules [6, 40] controversy exists in the literature concerning the role of these complexes in the binding of thrombospondin to platelets. Wolff et al. [41] reported that 10E5, a monoclonal antibody to GPIIb-IIIa, inhibited by up to 94% the binding of ^{125}I -thrombospondin to thrombin-stimulated platelets. However, they found [42] a normal binding of ^{125}I -thrombospondin and a normal expression of endogenous thrombospondin on thrombin-activated, GPIIb-

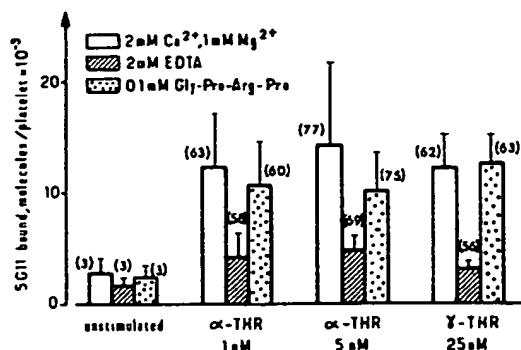


Fig. 3. Binding of 5G11 to platelets stimulated in the presence of EDTA or Gly-Pro-Arg-Pro. Platelets ($2.5 \times 10^8 \text{ ml}^{-1}$) were resuspended in Tyrode/albumin in the presence or absence of 0.1 mM Gly-Pro-Arg-Pro or in Tyrode/albumin with divalent cations replaced by 2 mM EDTA. Experimental conditions were as described in the legend to Table 1. ^{125}I -5G11 (20 $\mu\text{g/ml}$) was added for 30 min. Results are the mean \pm SD for eight experiments. The mean value for the release of 5-hydroxy[^{14}C]tryptamine is given in parentheses

IIIa-deficient platelets of two patients with Glanzmann's thrombasthenia. In the present study a saturating concentration of AP-2 failed to inhibit the surface expression of thrombospondin. However, although inhibiting by more than 85% the binding of ^{125}I -fibrinogen to platelets, AP-2 does not prevent the surface expression of platelet fibrinogen upon thrombin stimulation (Legrand, C., unpublished results). A similar finding for platelet fibrinogen has also been observed by Courtois et al. [43] using another antibody to GPIIb-IIIa complexes. Thus, these results do not preclude the possibility that thrombospondin is expressed through an interaction with fibrinogen bound to GPIIb-IIIa complexes at an early stage of the secretion process.

Fibrinogen and thrombospondin colocalize on the surface of activated platelets [12, 14]. The possibility that thrombospondin interacts with membrane-bound fibrinogen [16, 17] was supported by the fact that platelets stimulated in the presence of excess anti-fibrinogen Fab fragments bound approximately 50% fewer molecules of 5G11. However, we are unable to distinguish between a direct effect of the Fab fragments on thrombospondin binding to fibrinogen and a secondary effect on the membrane organization which would prevent thrombospondin binding to its receptor or reacting with 5G11 molecules. This could especially be so if the platelet receptor for thrombospondin is close to the receptor for fibrinogen as is implied from immunofluorescent and immunocytochemical studies [12, 14]. Furthermore, fibrinogen binding to GPIIb-IIIa complexes could modulate a different thrombospondin receptor in the membrane. A significant role for polymerizing fibrin in the thrombospondin expression could be excluded from the fact that appreciable 5G11 binding was observed after platelet stimulation with γ -thrombin.

For the above reasons, platelets from a patient with congenital afibrinogenemia deficient in platelet fibrinogen were studied. Here, binding of 5G11 to thrombin-stimulated platelets was at the lower end of the normal range. As the thrombospondin content of the platelets of this patient was somewhat below that usually observed, it is probable that a normal thrombospondin expression had occurred. This implies that when fibrinogen is lacking, thrombospondin expression can occur. Thus, there is a major fibrinogen-independent pathway for the binding of thrombospondin to platelets. Recent studies using the monoclonal antibody OKM5 have

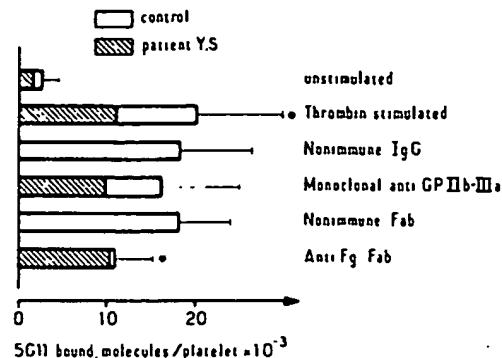


Fig. 4. Binding of 5G11 to platelets stimulated in the presence of various antibodies. Platelets ($2.5 \times 10^8 \text{ ml}^{-1}$) in Tyrode/albumin were stimulated for 3 min at 37°C with 1 nM α -thrombin in the presence of AP-2, a monoclonal antibody to GPIIb-IIIa complexes (20 $\mu\text{g/ml}$), polyclonal anti-fibrinogen Fab (100 $\mu\text{g/ml}$) or non-immune mouse IgG or rabbit Fab fragments. Hirudin was added and the platelets incubated for 30 min at room temperature with 20 $\mu\text{g/ml}$ ^{125}I -5G11. Results are the mean \pm SD for seven experiments. When performed, results for the platelets of patient Y.S. tested in parallel are also illustrated. * $P < 0.01$ Wilcoxon's rank sum test on paired samples

enabled Asch et al. [44] to propose that platelet membrane GPIV is a receptor for thrombospondin on platelets. This is a good candidate for the receptor for thrombospondin in the studies we report. Furthermore, GPIV is normally present in thrombasthenic platelets [5]. Binding of thrombospondin to GPIV may thus explain its normal binding to platelets deficient in GPIIb-IIIa and fibrinogen.

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Non-oncologic applications of radiolabeled peptides in nuclear medicine

L. C. KNIGHT

Radiolabeled peptides have been investigated for diagnostic imaging in a variety of nononcologic diseases. For imaging thromboembolic disease, peptides which bind to various components of thrombi have been tested. For targeting the fibrin component of thrombi, peptide analogues of fibrin or fragments of fibronectin which have a distinct binding domain for fibrin have been studied. For targeting activated platelets within thrombi, linear and cyclic peptide antagonists of the glycoprotein IIb/IIIa receptor on platelets have been studied, as well as naturally occurring antagonists of this receptor which are found in venoms. Analogues of laminin and thrombospondin which bind to other receptors on platelets have also been tested. There is an approach which uses a peptide to target thrombin which is sequestered within a fibrin clot. Another area of investigation has been to develop an improved radiopharmaceutical for imaging sites of infection and/or inflammation. Peptides which would bind to leukocytes *in vivo*, such as antagonists to the tuftsin receptor, chemotactic peptides, interleukin-8, or a platelet factor 4 analogue, have been radiolabeled for this purpose. These agents would enable imaging of both infection and inflammation. Development of a radiopharmaceutical for specifically imaging infection has focused on antimicrobial peptides such as human neutrophil defensin, ubiquicidin, human lactoferrin and alafosfalin, which are expected to bind selectively to microorganisms and not to leukocytes. Radiolabeled peptides are also being explored as agents for assessing unstable atherosclerotic plaque (endothelin), amyloid deposits (amyloid β

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peptides), and the consequences of diabetes mellitus (human C-peptide).

KEY WORDS: Peptides - Thrombosis - Infection - Inflammation - Arteriosclerosis - Diabetes mellitus - Amyloid.

One of the unique abilities of nuclear medicine is to noninvasively enable imaging of active biochemical processes *in vivo*. In many cases, altered processes in disease states are characterized by molecular targets which are more abundant in disease than in health. Radiotracers can be designed to recognize these targets and provide images which show the areas of increased uptake indicative of a disease process. This information can be used for initial diagnosis or to assess the progress of therapy.

A promising area of radiopharmaceutical development is the use of peptides as agents to carry a radionuclide to these targets. These peptides may be small analogues of larger proteins, such as native macromolecules which are normally involved in binding. Alternatively, targeting peptides may be found in natural products from other species, or they may be devised by structure-activity relationship studies to act as therapeutic antagonists of an unwanted process.

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In some cases, the physiologic target is a well-defined receptor. This enables design of carefully controlled studies to assess receptor binding *in vitro* and characterization of the behavior of a new radiolabeled peptide. In other cases, however, the exact target may not be well-defined and it may be difficult to distinguish nonspecific localization from specific targeting. Small peptides, if they do not exhibit binding to cells or proteins in the blood, tend to have relatively rapid blood disappearance. This may be due in part to this tendency, some experimental animal models may enable peptides to appear to bind to a lesion (e.g., a thrombus) when in actuality the peptide is accumulating nonspecifically in an area of edema due to an inflammatory process.

The use of radiolabeled peptides may provide improved imaging tests which provide advantages such as more rapid imaging results, greater ease of radiolabeling, and greater target specificity compared with current techniques.

Thrombosis

Deep vein thrombosis, or the formation of thrombi in the major veins of the extremities, remains a health problem affecting millions of people each year. In addition to the damage caused locally by the thrombi (postphlebitic syndrome), the thrombi may break loose and embolize to the lungs (pulmonary embolism) which can be fatal. Currently several tests in combination may be required to complete the diagnostic workup because each of the current tests has limitations. These tests include ultrasound examinations of the legs, blood tests for circulating D-dimer, VQ scans, spiral CT studies of the lungs and pulmonary angiograms. A radionuclide test which could rapidly locate both thrombi and emboli after a single injection could be very useful in assessing the need for anticoagulant therapy.

Venous thrombi are composed of layers of activated platelets alternating with layers of polymerized fibrin and numerous entrapped cells and plasma proteins.¹ The entrapped proteins include thrombin, which is an enzyme involved in both fibrin formation and platelet activation. Pulmonary emboli are composed of fragments of venous thrombi which, because their surface is thrombogenic, have become coated with new thrombus elements after they have

lodged in a pulmonary artery. If the embolus is exposed to high blood flow rates, the surface coating may be predominantly activated platelets. In contrast, if the embolus is completely occlusive the blood flow near it may be sluggish; this would probably lead to a fibrin-rich deposit on the surface of the embolus. For the purposes of targeting receptors in thrombi and emboli, therefore, it would seem that radiopharmaceuticals which recognize either activated platelets or fibrin would be suitable for imaging the lesions, as these components are present in high concentrations. Both of these approaches have been explored with radiolabeled peptides as targeting agents. Another approach has been to design a radiopharmaceutical which binds to molecules of thrombin which are trapped in the thrombotic lesion. This approach is more challenging because there are fewer sites to which a radiolabeled peptide could bind.

Figure 1 shows the amino acid sequences of some of the peptides which are being investigated for imaging thrombi and emboli.

Fibrin-targeted peptides

TP850 PEPTIDE FROM FIBRIN

Fibrinogen is a bivalent protein which contains 3 polypeptide chains on each half of the molecule (2 each of α , β and γ chains). During clotting, thrombin cleaves fibrinopeptides A and B from the N-termini of the α and β chains, respectively. The newly exposed domains at the N-termini of the α chain can bind to previously existing domains on the C-terminus of the molecule. The binding of these complementary domains leads to formation of a fibrin polymer.² The domain exposed by thrombin cleavage of fibrinopeptide A from the α chain has the sequence Gly-Pro-Arg-Val-Val-. Studies have shown that a peptide with a closely related sequence, Gly-Pro-Arg-Pro, is capable of inhibiting fibrin polymerization, presumably by binding to the polymerization site in the C-terminus of the γ chain of fibrin.³ The polymerization site to which this peptide binds is also present in fibrinogen, so the peptide would be expected to bind to circulating fibrinogen which is present in the blood at a concentration of approximately 8 μ M.

Gly-Pro-Arg-Pro-Pro was synthesized and tested as an agent for imaging thrombi. To label the peptide with ^{99m}Tc , a chelating moiety, Gly-DAla-Gly-Gly was added at the C-terminus, and a spacer, 4-amino-

Fibrin-targeted peptide	Fibrin-targeted peptides
TP850 GPRPP - Aba - GaGG	PAC-8 SYG RGD V RGD FKCTCCA
	Bitistatin
Thrombin-targeted peptide	SPVCGNEIL EQGEDCDCCS PANCQDQCCN
Hirulog ITPPGGGGDDGFEEIPEEYL	AATCKLTGPS QCNHGECCDQ CKFKKARTVC
	RIARGDWNDY YCTGKSSDCP WNH
	Laminin analogue
	CDPGYIGSR TP-1201 GaGG-Aba-YCSVTCG
	TP-1300 GaGG-Aba-YCSVTCR

Figure 1.—Examples of peptides under development for thrombus imaging. GaGG=Gly-DAla-Gly-Gly; Aba=4-aminobutyric acid; t=Dipic.

butyric acid (Aba) was incorporated in between to minimize steric hindrance.⁴ In rabbits with electrical-ly induced DVT (10 min old), γ camera images suggested thrombus uptake but there was no quantification of excised tissue specimens. The peptide was stable *in vivo* as judged by HPLC of urine specimens from rabbits. TP850 had rapid blood disappearance ($T_{1/2a}=4$ min, $T_{1/2b}=13$ min) and excretion was primarily renal.⁵ These findings suggest that there is minimal binding to circulating fibrinogen, which is a slow-clearing protein and would be processed by the liver.

FIBRIN-BINDING DOMAIN (FBD) OF FIBRONECTIN

Fibronectin is an adhesive protein which, among other functions, is involved in cross-linking of fibrin dimers. Controlled proteolysis of fibronectin yields fragments including a 31 kDa fragment from the amino terminus which contains both a fibrin-binding site and a site for covalent crosslinking of fibrin. This species was previously purified and labeled with ¹¹¹In for thrombus imaging.⁶ The fibrin-binding domain contains 5 repeated sequences, each of which contains 4 cysteine residues to constrain each subdomain into a loop so that the entire domain resembles the fingers of a hand. Smaller molecules containing 2, 3 or 4 of these loop subunits have been studied as potential thrombus-binding molecules. The smallest of these (2 loops, 12 kDa) has the fastest blood clearance and does not sacrifice binding activity, so it was investigated further. The 12 kDa fragment is produced by recombinant DNA expression. This molecule com-

petes effectively with endogenous fibronectin for binding to fresh clots, and binds to clots in the presence of heparin.

For clinical studies, 500 μ g of FBD protein are labeled with 20–25 mCi ^{99m}Tc by a direct stannous reduction technique (Draximage, Montreal). There was no report of whether the peptide was tested for disulfide bond cleavage after this procedure. *In vivo*, blood disappearance was fairly rapid: 20% remains after 30 min, 10% after 2 h and <2% after 24 h. The highest organ concentration is found in kidneys, followed by bladder, GI tract, liver, heart, lung and spleen.⁷ Clinically, imaging of the extremities is performed at several times up to 6 h postinjection so that 2 or 3 image sets from different time points can be compared. In an initial clinical trial of patients with DVT proven by compression ultrasound, the sensitivity was 80–93%.⁷

Platelet-targeted peptides

Human platelets express a number of receptors to which radiolabeled peptides can be directed. The most abundant receptor, as well as the most thoroughly studied, is the glycoprotein IIb/IIIa receptor (GPIIb/IIIa; also known as integrin α IIb β 3) which binds fibrinogen and is essential for platelet aggregation. There are approximately 50 000 to 100 000 copies of the receptor per platelet. GPIIb/IIIa has been studied as a target for antagonists to therapeutically prevent platelet aggregation in patients with coronary artery disease. Native ligands for this receptor include fibrinogen, vonWillebrand factor, fibro-

nectin, vitronectin and thrombospondin. Binding of these macromolecules can be inhibited by peptides containing the Arg-Gly-Asp (RGD) tripeptide motif. A number of synthetic peptides and peptidomimetics have been tested as antagonists for this receptor and some of these have also been explored as a basis for radiopharmaceuticals to bind to platelet deposits *in vivo*.

An important characteristic of platelets is their ability to become adhesive when stimulated, so that they can adhere to vessel wall and aggregate together to plug a leak in the vessel wall. This function is largely dependent on the GPIIb/IIIa receptor, and involves binding of adhesive macromolecules. For example, fibrinogen binds to the GPIIb/IIIa receptor and because it is bivalent, links 2 platelets together to achieve platelet aggregation. The GPIIb/IIIa receptor is not able to bind fibrinogen until the platelet is stimulated by an agonist such as ADP or thrombin. Studies with antibodies indicate that the number of GPIIb/IIIa receptors on the surface of the platelet does not change upon platelet activation/stimulation, but that the conformation of the receptor binding pocket may change to admit the binding domain of fibrinogen.

It should be noted that the native ligands for this receptor are all macromolecules which have a high degree of tertiary structure. Short linear peptides which represent a sequence from the domain of a larger native macromolecule will probably not have the optimal conformation to dock with the receptor with high affinity. The fact that many macromolecules contain —RGD— but bind to the GPIIb/IIIa receptor with different affinities supports the concept that the surrounding protein structure is important for determining the binding affinity of the receptor-binding domain. Furthermore, fibrinogen, which is the ligand with the highest affinity for GPIIb/IIIa, has multiple domains which are thought to interact with the receptor: 2 RGD domains and 1 HHLGGAKQAGIDV domain. Studies have shown that these domains bind sequentially, making the fibrinogen-receptor bond stronger (less likely to be reversible) with each additional contact point.

GPIIb/IIIa receptor ligands

LINEAR RGD CONTAINING PEPTIDES

These peptides have relatively low affinity for the GPIIb/IIIa receptor. A peptide derived from the antigen-binding domain of monoclonal antibody PAC-1 is

an example. The antibody was one of the few molecules found which was almost perfectly selective for binding to activated platelets over resting platelets. When the active binding region was sequenced, it was found to contain a peptide, ARRSPSYRYDGAPYYAMDY, which inhibited fibrinogen binding to platelets.⁸ Substitution of RGD for the RYD in this sequence improved the binding affinity for platelets. This peptide formed the basis for a family of RGD-containing peptides with up to 3 repeats of the RGD sequence. These linear peptides were modified for Tc labeling by inclusion of a KCTCCA metal-binding peptide sequence at either the N-terminal or C-terminal end of the peptide. These peptides were labeled by transchelation from ^{99m}Tc-glucuheptonate and tested *in vivo* in a canine model of 24-hour-old DVT.⁹ The peptides exhibited rapid blood clearance with predominantly renal excretion, and were apparently degraded by proteolysis. There was no detectable binding to circulating platelets. The percent of injected dose which bound to thrombus was low (0.006%ID/g); this was only slightly higher than negative control peptides (0.003%ID/g) or ^{99m}Tc-glucuheptonate (0.004 %ID/g).⁹ Images were characterized by diffusely increased uptake in the area around the thrombus and extensive soft tissue background. It appeared that the peptides were able to leak into the extravascular fluid and non-specifically accumulate because of edema in the tissue around the induced thrombus.

Investigators have shown that constraining the RGD sequence in a cyclic peptide greatly improves the affinity of the peptide for GPIIb/IIIa receptor.¹⁰ Cyclization has the added benefit of conferring increased resistance to proteolysis *in vivo*. The simplest way to achieve cyclization is to include 2 cysteines in the peptide and form a disulfide bond. This cyclization approach is not compatible with many technetium labeling approaches, however, as the reagents used to reduce the Tc can also reduce the disulfide bridge. Cyclization with a peptide bond or a thioether bond avoid this problem but require more synthetic expertise and cannot be done by many conventional peptide synthesis facilities.

P280

Peptide P280 is a 13 amino acid peptide comprised of a thioether-cyclized peptide including the pharmacophore [-Apc-Gly-Asp-] which is a mimetic for Arg-Gly-Asp. Apc stands for S-aminopropyl-L-cys-

teine, an Arg surrogate which confers additional selectivity for this receptor. The cyclic peptide is coupled to a Gly-Gly-Cys-Gly-Cys-Gly-Gly sequence for chelation of reduced Tc. The peptide (named bibapcptide) as supplied in a commercial kit (Diatide) is initially a dimer linked by a bis(succinimidomethyl) ether bridge through the 2 C-terminal cysteine residues but splits into 2 monomers during the labeling process. Compared to PAC-8 linear RGD peptide, P280 had much higher affinity for the GPIIb/IIIa receptor *in vitro*: $IC_{50}=79$ nM for P280 vs 12 000 nM for PAC-8 in an assay of inhibition of platelet aggregation in platelet-rich plasma. ^{99m}Tc -P280 was labeled by ligand exchange from ^{99m}Tc -glucoheptonate and tested in a standardized canine model of 24-h-old DVT. Like the linear ^{99m}Tc -PAC-8, it exhibited rapid blood clearance and very little binding to circulating platelets. The appearance of the images was similar as well: diffuse soft tissue activity and diffuse uptake in the muscle around the induced thrombus, probably a site of local edema. DVT binding was low (0.006%ID/g) with thrombus-to-blood ratios of 4.4 and thrombus-to-muscle ratios of 11.0 at 4 h postinjection.¹¹ In the same model, labeled autologous platelets exhibited thrombus binding of 0.18%ID/g, thrombus-to-blood ratios of 5.4 and thrombus-to-muscle of 230. In clinical trials, the biodistribution and clearance of ^{99m}Tc -P280 were similar to those observed in the canine model.^{12, 13} Anterior and posterior imaging of the lower extremities was carried out at 10, 60 and 120 minutes after injection. Based on 2 phase III clinical trials, sensitivities of 86-91% and specificities of 84-88% have been reported.^{14, 15}

DMP444

Peptide DMP444 is based on a cyclic peptide, DMP-728, that was the product of an extensive development effort to produce an antithrombotic therapeutic drug.¹⁶ The pharmacophore in these compounds is [(N-methyl-Arg)-Gly-Asp-] which confers high specificity for the GPIIb/IIIa receptor over other integrins.

DMP-444 also contains a HYNIC moiety for labeling the molecule with reduced technetium. Radiolabeling is accomplished with ^{99m}Tc -tricine and water-soluble phosphine coligands.¹⁷ When the radiotracer was tested in animal models of actively forming thrombi, the tracer was avidly incorporated into growing thrombi.¹⁸ Later studies of 24-h-old thrombi indicated that uptake of tracer was lower and throm-

bus-to-blood ratios were lower (2.3 for 24 h old vs 9.7 for 15 min old thrombi). ^{99m}Tc -DMP444 is excreted renally, with less than 0.3%ID excreted in the feces. This radiotracer appears to have slower clearance from the blood than P280.⁷ In initial clinical trials, anterior and posterior images of the lower extremities were obtained 1 h and 4 h after injection of ^{99m}Tc -DMP444. In a blinded comparison with compression ultrasound, sensitivity was 79% and specificity was 28%. These studies were used to improve image-reading criteria which led to improved sensitivity and specificity (93% and 75%) in subsequent patients.¹⁹

BITISTATIN

Bitistatin is a member of the disintegrin family of cysteine-rich polypeptides, many of which act as platelet aggregation inhibitors. Disintegrins were initially identified in viper venoms, but have since been found in other species as soluble proteins and in mammalian tissues. The molecules are highly folded into multiple loop structures and are constrained in this configuration by multiple disulfide crosslinks. Of the platelet-inhibitory disintegrins, all but one contains RGD at the apex of a flexible loop structure; this is believed to be the binding domain for GPIIb/IIIa.²⁰

A variety of disintegrins purified from viper venoms were radioiodinated and tested for their ability to image thrombi in the standardized canine model of 24-h-old DVT.²¹ Despite the sequence similarity among the group, distinct differences in thrombus uptake and biodistribution were observed. The disintegrin with the best imaging characteristics was bitistatin, isolated from *Bitis arietans* (puff adder) venom. Bitistatin contains 83 amino acids, with 7 internal disulfide crosslinks. ^{125}I -bitistatin had higher thrombus binding (0.210%ID/g) than any of the other disintegrins, although all disintegrins had better thrombus uptake (0.009-0.036%ID/g) than the short cyclic or linear peptides tested in the same model. At 4 h postinjection, ^{125}I -bitistatin thrombus-to-blood ratios (9.8) were higher than all other disintegrins and higher than positive controls ^{125}I -fibrinogen (2.8) and ^{99m}Tc -autologous platelets (5.0).²¹ ^{125}I -bitistatin displayed moderate blood clearance rates: faster than monoclonal antibodies, but slower than short synthetic peptides. A significant fraction of labeled bitistatin binds reversibly to circulating platelets *in vivo*. Excretion is primarily through the renal pathway. Besides the kidneys and bladder, the main organ visualized is the

spleen. Images of DVT were characterized by focal uptake which accurately reflected the size of the thrombus, and low soft tissue background for high thrombus-to-background ratios.

Bitistatin has been modified with HYNIC for labeling with reduced ^{99m}Tc . Labeling is accomplished by transchelation from ^{99m}Tc -glucoheptonate or ^{99m}Tc -tricine to avoid exposure of bitistatin's disulfide bonds to reducing agents.²² Bitistatin is now produced by recombinant DNA expression.²³

Labeled bitistatin was also tested for its ability to image pulmonary emboli (PE). In a model of 24-h-old PE, ^{125}I -bitistatin (0.64%ID/g) and ^{99m}Tc -bitistatin (0.89%ID/g) had higher uptake in PE than ^{125}I -fibrinogen (0.18%ID/g) or ^{99m}Tc -autologous platelets (0.14%ID/g). Ratios of PE-to-blood were also higher: 27 for ^{125}I -bitistatin vs 2.7 for fibrinogen and 4.1 for platelets. PE uptake of labeled bitistatin could be easily seen over lung background, and liver uptake is low.²²

Ligands for other receptors on platelets

LAMININ ANALOGUES

Laminin is a 900 000 Da basement membrane protein that promotes the attachment and migration of a variety of cells. The laminin receptor on platelets is present in about 1 000 copies per cell. This receptor is also known as VLA-6 or integrin $\alpha 6 \beta 1$. The sequence YIGSR from the center of the laminin chain has been identified as one of the main binding domains. A related peptide CDPGYIGSR was synthesized for testing as a thrombus-imaging agent.²⁴ The peptide was labeled with ^{99m}Tc by direct stannous reduction and was shown to bind to platelets. In normal rats, excretion was primarily through the kidneys with a small amount of hepatobiliary excretion.

THROMBOSPONDIN ANALOGUES

Thrombospondin (TSP) is a 420 000 Da protein which contains 3 identical subunits. It binds to fibronectin, laminin, type V collagen, and fibrinogen as well as to platelet surface receptors. Smaller analogues of thrombospondin were synthesized, based on the putative platelet binding domain of TSP, Cys-Ser-Val-Thr-Cys-Gly.²⁵ A related analogue which also has binding activity, Cys-Ser-Val-Thr-Cys-Arg, was also tested. Two peptides, TP1201 and TP1300 were generated from these starting sequences, coupled to a Tc binding sequence (Gly-DAla-Gly-Gly) and labeled

with ^{99m}Tc .²⁶ These peptides were capable of inhibiting platelet aggregation (IC50s were 337 and 306 nM for TP1201 and TP1300, respectively). In a rabbit model of PE, thrombotic uptake of these peptides was not as high as for the fibrin-targeted peptide ^{99m}Tc -P850 discussed above.⁵ Because of the differences between the animal models, the *in vivo* behavior of these peptides could not be directly compared with other platelet-binding radiotracers discussed above.

Thrombin-targeted peptides

There have been attempts to image thrombi by using a radiopharmaceutical which binds to thrombin remaining in the fibrin network of a thrombus. Normally thrombin in the blood is generated from its zymogen, prothrombin, as needed. Excess thrombin in the blood is quickly neutralized by endogenous inhibitors such as antithrombin III. It is theorized that thrombin in a clot is protected from such inhibition, but is accessible to peptide radiotracers which could bind there.

HIRUDIN ANALOGUES

Hirudin is a 65 amino acid thrombin inhibitor found in the saliva of the leech *Hirudo medicinalis*. This single chain peptide contains 3 intra-chain disulfide bridges and a sulfated tyrosine. It binds to both exosite I of thrombin (the fibrin binding site) as well as to the active site of thrombin. Recombinant hirudin has been produced but is not sulfated at the tyrosine residue. Hirulog (bivalirudin, BG8967; Biogen, Cambridge, MA, USA) is a synthetic 20 amino acid peptide rationally designed from structural studies of hirudin. It is an approved thrombin inhibitor for the management of cardiovascular disease, as an alternative to heparin. Hirulog contains 2 binding domains. The amino terminal domain, DPhe-Pro-Arg-Pro-, binds to the active site of thrombin. It is joined by a 4-glycine linker to the 2nd domain DGDFFETPEFYI, which is modeled after the carboxy terminus of hirudin. This 2nd domain binds to the anion-binding exosite in thrombin (the fibrinogen binding site of thrombin). Hirudin and hirulog are believed to inhibit clot-bound heparin. In contrast to hirudin, which binds tightly to thrombin, hirulog is transient inhibitor of thrombin.²⁷ The Arg-Pro bond in the amino terminal domain is readily cleaved, resulting in a molecule with weaker inhibitory activity.²⁸

Hirulog was labeled with ^{125}I using the Chloramine-T method. The labeled peptide was initially evaluated in

a rat thrombus model in which an etched metal thread was used to initiate thrombus formation. Problems with the instability of the radioiodine label need to be solved for creation of an optimized imaging agent.²⁹

Inflammation/infection

A recent article provided an excellent review of all the current radiopharmaceuticals for imaging inflammation and infection.³⁰ These conditions are often discussed together, perhaps because they have been difficult to differentiate by the imaging tests which have been available. Inflammation is the reaction of the body to injury, whether or not it involves microorganisms. Infection is the result of invasion by microorganisms. Because white blood cells invade both types of lesions, labeled white blood cells can detect both inflammation and infection. It is generally recognized that autologous white blood cells labeled *ex vivo* with ¹¹¹In-oxine or ^{99m}Tc-HMPAO are useful for imaging inflammation and infection, but that the labeling method is not convenient. The radiolabeled peptides discussed in this section are primarily based on an attempt to label white blood cells *in vivo* by a simple injection of radiolabeled peptide.

TUFTSIN ANALOGUES

RP128 is an asymmetrical chelator-containing antagonist to the tuftsin (immunomodulating peptide) receptor. The tuftsin receptor is expressed by neutrophils, monocytes and macrophages. Tuftsin is a leukokinin-derived tetrapeptide that promotes chemotaxis of these phagocytic cells. An antagonist peptide, TKPRR, has 4-fold greater receptor affinity than does tuftsin. TKPRR was coupled *via* a glycine residue to dimethyl-Gly-Ser-Gly (acetamidomethyl), an N3S chelator for ^{99m}Tc, to create RP128. RP128 (unlabeled) was able to compete with iodinated VEGF-A165 for binding to HUVEC, with an IC₅₀ of 11.5 μ M.³¹ In animal models, ^{99m}Tc-RP128 accumulates in both infections and sterile inflammation. In human subjects, ^{99m}Tc-RP128 cleared rapidly from blood and other tissues, with no significant accumulation except kidney and bladder and the synovia of some joints, especially the knees. The low background in the abdomen should be favorable for imaging Crohn's disease. The peptide accumulated in clinically affected joints in rheumatoid arthritis although no gold standard was available for

comparison. The peptide did not bind significantly to cells in whole blood, in contrast to expected binding to neutrophils. The mechanism of uptake therefore remains to be explained. It may be that the number of receptors on resting neutrophils is low in contrast to activated neutrophils.³²

CHEMOTACTIC PEPTIDES

Formyl-Met-Leu-Phe (f-MLF) is a chemotactic peptide which binds to white blood cells. It has been studied for a number of years as a way to carry a radiolabel to neutrophils, using a variety of labeling approaches. There is concern about the neutropenia caused by administering even low doses of this chemotactic peptide. It is not clear whether the specific activity of the labeled peptide can be made high enough to avoid neutropenia.

TP765 is an analogue of fMLF which contains a tetrapeptide Gly-Gly-DAla-Gly to complex reduced ^{99m}Tc. The tetrapeptide chelator is placed on the carboxy terminus of the chemotactic peptide, with an intervening spacer (4-aminobutyric acid) to minimize steric effects. In animals, ^{99m}Tc-TP765 cleared rapidly from the blood (90% had cleared by 45 minutes) and accumulation was seen in sterile abscesses and sites of bacterial infection.

A related chemotactic peptide, fMLFK, was labeled with ^{99m}Tc using ethylene dicysteine (EC) as chelating system to enhance renal excretion. In a preformed chelate approach, EC was labeled with ^{99m}Tc, activated at a carboxy function and coupled to the peptide. When added to blood, 68.1% of activity was bound to WBC, of which 86% was bound to granulocytes. In mice, however, excretion was predominantly hepatobiliary (24.5 \pm 48.8%) not renal (17.9%). Because of the resultant abdominal activity, this peptide was judged unsuitable for imaging infections.^{33, 34}

S-benzyl mercaptoacetyl dipeptides containing 0, 1 or 2 carboxyl functions were used to derivatize fMLFK for labeling with reduced Tc. Although inclusion of carboxyl functions reduced hepatobiliary excretion, the reduction was not adequate to make these peptides suitable for *in vivo* imaging of occult infection in the abdomen.³⁴

INTERLEUKIN-8 (IL-8)

IL-8 is a 72 amino acid chemotactic cytokine (Figure 2) that binds to CXC type I and type II receptors on

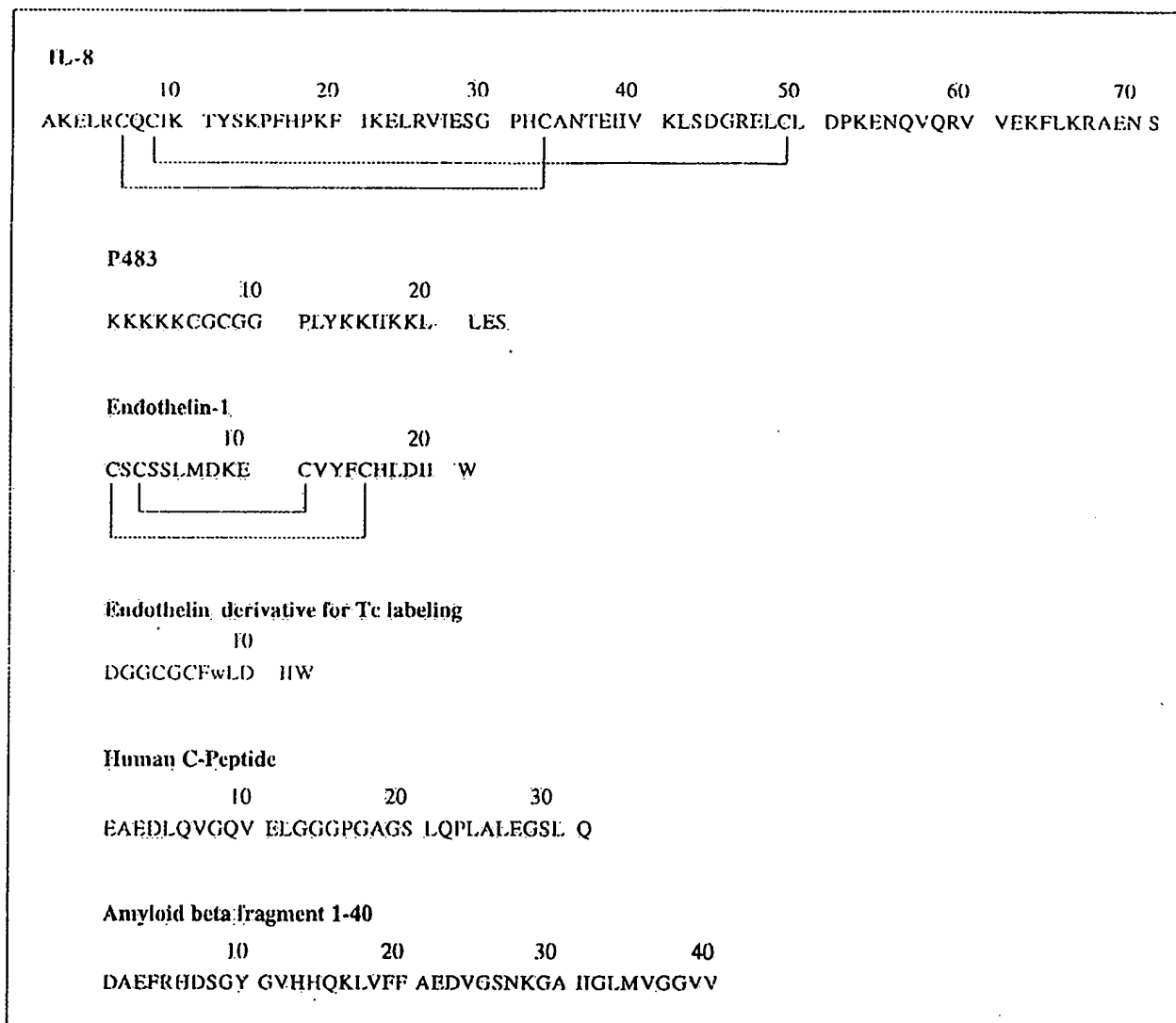


Figure 2. Other peptides. w=DTp. Lines indicate disulfide bridging pattern.

neutrophils and monocytes with high affinity (0.3-4 nM). These receptors are present in high concentrations (20 000-80 000/neutrophil). A concern with the use of this peptide is its effect on circulating leukocyte counts: a dose of 25 µg/kg causes a transient drop in leukocyte counts. Recombinant IL-8 was radioiodinated with ¹²⁵I for initial clinical studies ³⁵ but the specific activity was not high enough to avoid transient neutropenia.

Recombinant IL-8 was then labeled with ^{99m}Tc using a HYNIC chelating agent. In rabbits with *E. coli* thigh infection, ^{99m}Tc-IL-8 performed better than a negative control (^{99m}Tc-lysozyme): uptake (0.33% vs 0.025% ID/g), abscess:blood (11.9 vs 0.24) and abscess:muscle (127 vs 6.7). ^{99m}Tc-HYNIC-IL-8 is excreted renally with 28%ID retained in kidneys. Neutropenia was transient (30 min) and moderate.³⁶ In rabbits with pulmonary infections (aspergillosis in immunocompromised rab-

bits or *E. coli* induced pneumonia in immunocompetent rabbits), ^{99m}Tc -HYNIC-IL 8 was superior to ^{67}Ga -citrate with respect to target-to-background ratios, lower background signal and lower radiation exposure.³⁷

When recombinant IL-8 was labeled with ^{99m}Tc using the HYNIC bifunctional chelator, it was found that the properties of the labeled peptide varied greatly depending upon the coligand used to stabilize the Tc-HYNIC complex. Of various coligands tested with this system, tricine+nicotinic acid resulted in the best properties for infection imaging: high specific activity (up to 80 MBq/ μg), high *in vitro* stability; high abscess/muscle ratios (>200) and high abscess/background ratios (>20) in rabbits with *E. coli* infections. The high specific activity label would permit imaging with administered doses of peptide as low as 70 ng/kg.³⁸

PLATELET FACTOR 4 ANALOGUE

P483 is a peptide derivative of the heparin binding region of platelet factor 4. The sequence is given in Figure 2. ^{99m}Tc -P483H is a peptide-heparin complex. This peptide complex binds to monocytes and neutrophils, as determined by microscopic studies of fluorescent binding. Binding to these cells could be reduced by 100-fold excess cold peptide.³⁹ In rabbits with 24-h acute *E. coli* muscle infections, ^{99m}Tc -P483H had higher infection/blood (2.5) and infection/muscle (23.5) than other common tracers (0.2-1.8 and 6.1-15.4) such as BW 250/183, LeukoScan, TechnoScan, RP128 and human WBCs. In a model of inflammation (human white blood cell depot in rabbit muscle) lesion/blood and lesion/muscle ratios (1.6 and 4.9, respectively) were lower in inflammation than in infection.⁴⁰

Infection-selective agents

It would be desirable to have an imaging test which could differentiate between infection and sterile inflammation.⁴⁰ The peptide approach to this has involved labeling antimicrobial peptides, which specifically bind to microorganisms rather than white blood cells. The amino acid sequence of a number of these peptides is given in Figure 3.

DEFENSIN

Human neutrophil peptide-1 (HNP-1) is a member of the defensin family of mammalian antimicrobial

peptides. This 30 amino acid peptide contains 3 intermolecular disulfide bridges. It is stored in the granules of human neutrophils and kills microorganisms after they have been phagocytized. The killing mechanism is thought to be related to an electrostatic interaction between the positively charged defensin peptide and the negatively charged bacterial surface molecules, rendering the bacterial cell wall permeable. HNP-1 was obtained from human neutrophil granules and purified by reversed-phase HPLC. The peptide was labeled with ^{99m}Tc by a direct stannous chloride/potassium borohydride method. It is not known whether the disulfide bridges were altered by this process. ^{99m}Tc -HNP-1 accumulated at sites of bacterial infection in mice faster than ^{99m}Tc -IgG. However, ^{99m}Tc -HNP-1 appeared to wash out from the infection after reaching peak uptake at 15 min. The explanation for this reversal may be the antibacterial effect of the labeled peptide. ^{99m}Tc -HNP-1 does not bind significantly to neutrophils but binds to bacteria.⁴¹

^{99m}Tc -HNP-1 had higher discrimination for bacteria over leukocytes than ^{99m}Tc -ubiquitin peptides (see below), when tested in a mouse model.⁴² It is not preferred over ubiquitin peptides, however, because it is too large and complex to be conveniently prepared synthetically.

UBIQUITIN ANALOGUES

UBI is a 6.7 kDa linear peptide (59 amino acids) which is a natural mammalian antimicrobial agent. Synthetic peptides comprising various portions of UBI were synthesized and tested for their ability to target bacterial infections.⁴² Each peptide was radiolabeled with ^{99m}Tc by a direct reduction technique.⁴¹ It is not known which amino acids in the peptides bind the reduced Tc or how this affects the structure of the complex. The labeled peptides were stable in serum *in vitro* and >90% was excreted intact in urine collected from experimental animals.⁴³

In vitro, ^{99m}Tc -UBI peptides showed higher binding to bacteria than to leukocytes. The peptides with the best binding to bacteria were UBI 18-35, UBI 31-38, UBI 29-41 and UBI 22-35. The degree of binding did not correlate with the number of positive charges on the peptide. Binding of these peptide radioligands could be reduced by preincubating the cells with unlabeled corresponding peptide, which suggests a specific interaction with the bacteria. Interestingly, binding of ^{99m}Tc -intact UBI (1-59) to bacterial cells *in*

vitro was much lower than the binding of the labeled short peptides.⁴²

^{99m}Tc-UBI peptides were tested for their ability to detect bacterial infections and distinguish them from sterile inflammations. In mice and rabbits with *S. aureus* or *Klebsiella pneumoniae* infections, images of sites of infection with ^{99m}Tc-UBI 18-35 and ^{99m}Tc-UBI 29-41 were positive by 5-15 min postinjection.⁴⁴ Target/nontarget ratios of up to 5 were obtained by 4 h.⁴⁵ In *S. aureus* infection, the uptake of ^{99m}Tc-UBI 18-35 and ^{99m}Tc-UBI 29-41 correlated with the bacterial density, measured before and after antibiotic treatment.⁴⁶ No significant accumulation of tracer (target/nontarget <1.5) was seen in animals with sterile inflammations.⁴⁴ Interestingly, Tc-UBI did not detect sterile inflammation caused by injection of heat-killed bacteria, despite the proposed mechanism of binding electrostatically to bacterial cell wall.⁴⁵

^{99m}Tc-UBI 29-41 was able to detect *S. aureus* infections in mice even if the mice were leukocytopenic, indicating that the mechanism of localization in infection does not involve leukocytes.⁴⁵ Studies comparing ^{99m}Tc-UBI 29-41 with a scrambled-sequence version of the peptide indicated that both bind to bacteria, but the scrambled sequence bound less well. It may be that the antimicrobial function of UBI peptides is based both on amino acid sequence and the cationic character of the peptide.⁴³

In vivo biodistribution studies in mice showed that excretion of ^{99m}Tc-UBI 29-41 is rapid and primarily through the kidneys.⁴³

HLF PEPTIDES (HUMAN LACTOFERRIN PEPTIDES)

Lactoferrin is a 692-amino acid iron-binding protein found in body fluids, secretory granules of neutrophils and mucosal epithelium. It inhibits viral and bacterial growth, and it has bactericidal action related to its ability to bind to bacterial membranes and disrupt them. Short peptides which contain highly cationic domains near the N-terminus have been found to have antimicrobial activity without the iron-binding function.⁴⁷

Short peptides from lactoferrin (Figure 3) were tested for their ability to target infections. The peptides hLF 1-11 and 2-11, when labeled with ^{99m}Tc by a direct reduction technique, bound well to bacterial cells *in vitro*. Unlike ^{99m}Tc-UBI peptides, however, the labeled hLF peptides also bound to human leukocytes. This makes them less useful for imaging infection, because they cannot adequately discriminate between bacte-

rial infection and sterile inflammation. Furthermore, the peptides tested had a relatively high degree of hepatobiliary clearance.⁴²

ALAFOSFALIN

Alafosfalin, (S)-alanyl-(R)-1-aminoethylphosphonic acid, is a dipeptide phosphonic acid. It has broad-spectrum antibiotic activity, and is actively transported into the intracellular space of bacterial cells by stereospecific peptide permeases, after which it is cleaved intracellularly to a metabolite which binds to and inhibits alanine racemase and interferes with bacterial cell wall synthesis. The compound is labeled with ^{99m}Tc by a direct reduction technique. It is hypothesized that the Tc is coordinated with both amino nitrogens and the phosphate hydroxy group, possibly with 2 alafosfalin molecules per Tc. It is believed that the functional groups needed for binding to the permease protein are used for binding the metal. Despite this, ^{99m}Tc-Alafosfalin was shown to bind to *S. aureus* and binding was reduced with increasing concentrations of peptide. In rats, the radiolabeled peptide cleared rapidly from blood and was concentrated primarily in kidneys/urine and bone. Very little liver or spleen activity was seen. There was considerable diffuse muscle background in images. The peptide accumulated in *S. aureus* infection in the thigh of rats, although the abscess concentration declined between 1 and 4 hours whereas ^{99m}Tc-HMPAO-leukocytes (positive control) continued to accumulate during this time. The target-to-nontarget ratio of 4.3 at 4 h was lower than Tc-leukocytes (20 at 4 h).⁴⁸

Other diseases

Atherosclerosis

ENDOTHELIN

Endothelin is a 21 amino acid peptide (Figure 2), is a potent vasoconstrictor which helps to maintain vascular tone by interacting with receptors ETA and ETB. Alteration in endothelin function is involved in various vascular diseases. In human atherosclerotic coronary arteries, high densities of ETB receptors colocalize with infiltrating macrophages. It may be that the density of macrophages in atherosclerotic lesions is an indicator of plaque vulnerability and the risk of plaque rupture.

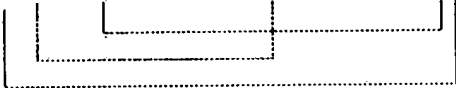
<u>Ubiquicidin-related peptides</u>		
UBI 1-18	KVIHGLARAGKVRGQTPK	
UBI 18-29	KVAKQEKKKKKT	
UBI 29-41	TGRAKRRMQYNRR	
UBI 18-35	KVAKQEKKKKKTGRAKRR	
UBI 22-35	QEKKKKKTGRAKRR	
UBI 31-38	RAKRRMQY	
<u>Human lactoferrin-related peptides</u>		<u>Defensin HNP-1</u>
HLF 1-11	GRRRRSVQWCA	
HLF 2-11	RRRRSVQWCA	
HLF 21-31	FQWQRNMRKVR	
		10 20 30
		ACYCRIPACI AGERRYGTCI YQGR LWAFCC
		

Figure 3.—Antimicrobial peptides. Lines indicate disulfide bridging pattern.

The peptide Endothelin-1 was labeled using 4-[¹⁸F] fluoro benzoate and purified by reversed-phase HPLC. The radioligand was found to bind *in vitro* to renal medulla but had only low binding to renal cortex. Binding to medulla was blockable with unlabeled endothelin-1 and partially by ligands specific for ETA and ETB.⁴⁹ *In vitro*, high levels of ¹⁸F-endothelin binding to coronary artery plaque were found co-localised with infiltrating macrophages. This radioligand may be useful for PET imaging of vulnerable coronary artery plaques.⁵⁰

A smaller endothelin derivative was created which contains the binding sequence Phe-D-Trp-Leu-Asp-Ile-Ile-Trp based on the C-terminal sequence of endothelin-1. It was synthesized to contain a Tc-binding sequence, Asp-Gly-Gly-Cys-Gly-Cys, at the N-terminus. After labeling with ^{99m}Tc, this peptide was tested for its ability to localize in atherosclerotic aortas in rabbits. Scintigraphy was positive for radiotracer uptake within 15 minutes after injection. Histologic analysis of the aorta indicated that accumulation of this radiotracer correlated with the number of neointimal smooth muscle cells. Radiotracer accumulation was also noted in the liver, heart (blood pool) and kidneys.⁵¹

Diabetes

C-peptide (Figure 2) is a connecting peptide which is removed from the precursor of insulin (proinsulin)

during the release of the mature insulin molecule. C-peptide may be useful in preventing or limiting some of the complications of diabetes because of its actions on vascular and nervous systems.⁵² C-peptide has been shown to bind to cell membranes, bind eNOS and Na+K+-ATPase activity and to affect renal and nerve dysfunction in insulin-dependent diabetes patients. C-peptide was labeled by conjugating the N-terminus to N-succinimidyl-[¹⁸F]fluoro benzoate. In a monkey model, excretion was primarily by the renal pathway. The goal of this study was limited to studying normal tissue distribution prior to performing studies in patients.⁵³ It is not yet known how a labeled C-peptide analogue might be used in providing clinical diagnostic information.

Amyloid deposits/Alzheimer's disease

Amyloid deposits are associated with a variety of diseases including Alzheimer's disease, type II diabetes mellitus and dialysis arthropathy. Amyloid deposits take the form of fibrillar aggregates which interfere with normal tissue growth and function. Amyloid deposits apparently are constantly being turned over, because when treatment is provided to stop the formation of new fibrils, the existing deposits regress.⁵⁴ Serum amyloid P component, a plasma protein, has been labeled with ¹²⁵I and ¹³¹I and used

to image systemic amyloidosis clinically.⁵⁵ It has not been successful for imaging Alzheimer's disease, however, perhaps because its large size prevents it from crossing the blood/brain barrier.⁵⁶

Radiolabeled peptides are under development for imaging amyloid deposits in Alzheimer's disease. Beta-amyloid deposits can form from polymerization of β -amyloid peptide fragments from proteolysis of amyloid precursor protein, so labeled peptide fragments should be incorporated into amyloid fibrils.

¹²⁵I-labeled amyloid β (1-40) (Figure 2) is able to cross the blood-brain barrier to reach amyloid deposits in brain. For external imaging, an analogue of amyloid β (1-40) was synthesized with a DTPA group replacing the 2 N-terminal amino acids. This peptide conjugate was labeled with ¹¹¹In. ¹¹¹In-DTPA-amyloid β (3-40) had the same binding rate and extent to *in vitro* amyloid deposits as ¹²⁵I-amyloid β (1-40), and was able to bind to and image synthetic amyloid deposits in rats. The peptide was stable in rat blood for several hours. The binding was judged to be specific by autoradiographic comparison with tissue stains.⁵⁷

The amino acid sequence essential for β -amyloid peptide polymerization has been identified to be KLVFF, which binds to other identical domains in other amyloid peptide strands. A synthetic peptide, ffvfk, which uses all D-amino acids and the reverse sequence (retro-inverso) has twice the affinity of the native pentapeptide sequence. This peptide was used to prepare a tandem dimer linked at the 2 C-termini by a Lys- β -Ala spacer. It was also used to produce a hexamer based on 8-branched amino-PEG (the remaining 2 amino groups were coupled to biotin; final MW=10 kDa). The peptides and conjugates were labeled with either tritium or a fluorescent tag for detection. The tandem dimer had 100-fold greater affinity for β -amyloid fibrils *in vitro* than the monomer, and the 6-copy ffvfk on a PEG backbone had a 10 000-fold greater affinity (K_d=0.1 nM). A positively charged group is essential to inhibit fibril formation. A remaining problem is to radiolabel this conjugate and enable it to cross the blood-brain barrier.⁵⁸

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2003 IPA RESEARCH AWARDS FIRST-PRIZE WINNER

The Rowland Universal Dementia Assessment Scale (RUDAS): a multicultural cognitive assessment scale

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ABSTRACT

Objective: To develop and validate a simple method for detecting dementia that is valid across cultures, portable and easily administered by primary health care clinicians.

Design: Culture and Health Advisory Groups were used in Stage 1 to develop culturally fair cognitive items. In Stage 2, clinical testing of 42 items was conducted in a multicultural sample of consecutive new referrals to the geriatric medicine outpatient clinic at Liverpool Hospital, Sydney, Australia ($n = 166$). In Stage 3, the predictive accuracy of items was assessed in a random sample of community-dwelling elderly persons stratified by language background and cognitive diagnosis and matched for sex and age ($n = 90$).

Measurements: A research psychologist administered all cognitive items, using interpreters when needed. Each patient was comprehensively assessed by one of three geriatricians, who ordered relevant investigations, and implemented a standardized assessment of cognitive domains. The geriatricians also collected demographic information, and administered other functional and cognitive measures. DSM-IV criteria were used to assign cognitive diagnoses. Item validity and weights were assessed using frequency and logistic regression analyses. Receiver-operating characteristic (ROC) curve analysis was used to determine overall predictive accuracy of the RUDAS and the best cut-point for detecting cognitive impairment.

Results: The 6-item RUDAS assesses multiple cognitive domains including memory, praxis, language, judgement, drawing and body orientation. It appears not to be affected by gender, years of education, differential performance factors and preferred language. The area under the ROC curve for the RUDAS was 0.94

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(95% CI 0.87–0.98). At a cut-point of 23 (maximum score of 30), sensitivity and specificity were 89% and 98%, respectively. Inter-rater (0.99) and test-retest (0.98) reliabilities were very high.

Conclusions: The 6-item RUDAS is portable and tests multiple cognitive domains. It is easily interpreted to other languages, and appears to be culturally fair. However, further validation is needed in other settings, and in longitudinal studies to determine its sensitivity to change in cognitive function over time.

Key words: dementia, multicultural, cognition, assessment, culturally fair, primary health care

Introduction

Projected increases in migration and the aging population will have significant demographic and social consequences for many of the world's countries during the first half of the twenty-first century (Martin, 2001; United States Department of Commerce *et al.*, 1999). In 2001, approximately 150 million people lived outside their country of birth (Martin, 2001). By 2025, the world's population 65 years of age and above will more than double, and economically, the world community will face an elderly support burden almost 50% larger than that in 1998 (United States Department of Commerce *et al.*, 1999).

Disorders such as dementia, which disproportionately affect the oldest age groups, cause considerable morbidity to patients and carers, and generate large health-care costs. Early detection of dementia is critical for the purposes of differential diagnosis, secondary prevention and psychosocial intervention (Brodaty and Moore, 1997; Sandson and Price, 1996; Small, 1998). Developing a simple method for detecting dementia that is valid across cultures, easily portable and easily administered by primary health care clinicians would provide more equitable access to health care to those already at high risk of poor outcomes.

Most previous attempts to develop instruments for this use in culturally diverse populations modified or translated scales that were originally developed in dissimilar groups. For example, the Folstein Mini-Mental State Examination (MMSE) was developed in an English-speaking population, but is nevertheless commonly used to assess cognition in persons from culturally and linguistically diverse backgrounds (Folstein *et al.*, 1975). MMSE scores are influenced by age, education, ethnicity and language of the interview (Escobar *et al.*, 1986). Many words cannot be easily translated and several concepts are not relevant to people from other cultures. On excluding items that might be culturally biased, ethnic differences in the rates of "severe" cognitive impairment disappeared. Escobar concluded that the MMSE should be revised to diminish social and education artefacts, through item selection and weighting. Furthermore, the

MMSE usually fails frontal lobes (Royall

The Fuld Object develop an instrument is not easily portable standardize across cultures. For African Americans, the FOM (Mast *et al.*, 2001).

The Cognitive Assessment Scale (CAS), the MoCA, the Screening Scale (Folstein *et al.*, 1992). C of standardized item effect for education (2001).

The Elderly Cognitive Function Scale (Singapore in a predictive study of the MMSE and the 1976; Folstein *et al.*, to test memory recall date of birth) that is valid across backgrounds. The 91.5%.

The 2-stage Cross-Cultural Cognitive Examination (CCCE) for use in cross-cultural settings. The CCCE was developed using the translation and adaptation reported 100% sensitivity and the 94% sensitivity was impressive. However, the study was 52.6 years old and demented. The validity and the results may be affected.

The Community Cognitive Examination (CCE) is the translation-based informant-based instrument used in Manitoba, and the CSF'D is further revised. A revised version of the CCE for African Americans in Nigeria (Hall *et al.*,

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MMSE usually fails to detect cognitive impairment primarily involving the frontal lobes (Royall *et al.*, 1994).

The Fuld Object-Memory Evaluation (FOME) was an early attempt to develop an instrument that was culturally fair (Fuld *et al.*, 1988). This instrument is not easily portable, tests only a few cognitive domains, and is difficult to standardize across cultural settings. In a study of elderly African and European Americans, the FOME had a sensitivity of 93%, but a specificity of only 63.5% (Mast *et al.*, 2001).

The Cognitive Abilities Screening Instrument (CASI) borrowed items from the MMSE, the Modified Mini-Mental State Test and the Hasegawa Dementia Screening Scale (Folstein *et al.*, 1975; Hasegawa, 1983; Teng and Chui, 1987; Teng *et al.*, 1992). Comparisons across cultural groups are difficult, due to a lack of standardized items in multiple versions of the instrument and the significant effect for education in some cultural groups (Graves *et al.*, 1993; Shadlen *et al.*, 2001).

The Elderly Cognitive Assessment Questionnaire (ECAQ) was developed in Singapore in a predominantly male population, and includes 10 items taken from the MMSE and the Geriatric Mental State Schedule (GMS) (Copeland *et al.*, 1976; Folstein *et al.*, 1975; Kua and Ko, 1992). The ECAQ uses a 4-digit number to test memory recall, and relies heavily on demographic information (such as date of birth) that may be difficult to confirm in patients from diverse cultural backgrounds. The ECAQ reports a sensitivity of 85.3% and specificity of 91.5%.

The 2-stage Cross-Cultural Cognitive Examination (CCCE) was developed for use in cross-cultural epidemiological dementia research (Glosser *et al.*, 1993). The CCCE was developed in subjects from Guam and the United States, and used the translation-back-translation procedure to ensure cultural fairness. The reported 100% sensitivity and 83% specificity of the 5-minute, 6-item screen and the 94% sensitivity and 99% specificity of the 20-minute mental state exam are impressive. However, the average age of the participants in the Guam validation study was 52.6 years, and only 18 of 115 were diagnosed as definitely or possibly demented. The young age of the participants is unusual in dementia research, and the results may be difficult to generalize to other older populations.

The Community Screening Interview for Dementia (CSI'D) also used the translation-back-translation procedure to develop a lengthy cognitive and informant-based instrument for use in Cree-speaking natives living on reserves in Manitoba, and English-speaking residents in Winnipeg (Hall *et al.*, 1993). The CSI'D is further limited by the need to interview a reliable informant. A revised version was recently evaluated in the original samples as well as in African Americans in Indianapolis and Jamaica and the Yoruba population in Nigeria (Hall *et al.*, 2000). The areas under the receiver-operating characteristic

(ROC) curve with and without informant data were 0.82–0.97 and 0.74–0.93, respectively. Education had a significant effect at most sites.

The Mini-Cog is the shortest and most portable of the recent cross-cultural instruments (Borson *et al.*, 2000). It combined a 3-item memory task and a clock drawing task, and reported a sensitivity of 99% and a specificity of 93% in the development sample (validation data not reported). While the 3-item recall task was the more powerful component in the Mini-Cog, our work suggests that the accuracy of clock drawing may be modest at best in multicultural samples (Storey *et al.*, 2002).

Researchers who believe that behavior can only be understood within the cultural context in which it occurs suggest that concepts and instruments should be developed for each distinct cultural group (Kim, 2000). Can we reasonably believe however, that Italians who migrated to Australia 50 years ago are similar culturally and linguistically to those who have always lived in Italy? Acculturation research suggests that the same criteria should not be used to compare natives living in their country of cultural origin to those who have relocated to a different culture (Berry, 1997; Bourhis *et al.*, 1981; Harwood *et al.*, 1994). Furthermore, the existence of several thousand different cultures and languages makes the development of distinct instruments for each group clearly impractical (Bernatzik, 1957; Swadesh, 1967). While we acknowledge that both inherited and environmental factors probably influence cognition, we believe that humans have common experiences that allow cognition to be measured independent of cultural differences (Berry *et al.*, 1992).

In this paper, we report the development and validation of the Rowland Universal Dementia Assessment Scale (RUDAS), a scale that fulfils many of the requirements needed to accurately assess cognition in culturally diverse populations.

Method

Stage 1 – Development of Items

AIMS

The aims of the item-development stage were to:

1. Identify cognitive domains important in the assessment of cognition (particularly those associated with early cognitive impairment).
2. Propose potential items to measure cognition in each of these domains.
3. Optimize the psychometric validity, and cultural and linguistic equivalence of the proposed items.
4. Develop a final list of items to test in a culturally heterogeneous population.

Table 1. Aims and our

GROUP	AIM
1st Health	Identify in assess:
1st Culture	Explore Assess c cognitiv relevanc sensitivi normal
2nd Health	Propose nitive d and vali based it
2nd Culture	Assess and lin modific
3rd Health	Revise items.
3rd Culture	Final review.

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ITEM-INCLUSION
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Table 1. Aims and outcomes of advisory groups in item development

GROUP	AIM	OUTCOME
1st Health	Identify cognitive domains important in assessment of cognition.	List of domains, including memory, learning, attention, orientation, language, gnosis, visuo-spatial, perseveration/initiation, praxis, planning, judgment, insight, function and behavior.
1st Culture	Explore the meaning of culture. Assess cultural relevance of proposed cognitive domains for conceptual relevance and importance, cultural sensitivity and specificity, and role in normal and abnormal function.	All domains endorsed as culturally relevant.
2nd Health	Propose potential items for each cognitive domain (previously published and validated items, and experiential-based items).	List of approximately 80 items.
2nd Culture	Assess proposed items for cultural and linguistic fairness, and suggest modifications where appropriate.	Refined list of items (some discarded, some modified).
3rd Health	Revise validity of culture modified items.	Validity-based revised list of items.
3rd Culture	Final cultural and linguistic item review.	List of 60 items for clinical testing.

ADVISORY GROUPS

After a comprehensive review of the literature, we formed two advisory groups to help us identify and appraise potential cognitive domains and items. The Health Group included professionals from a number of health disciplines (geriatric medicine, aged care psychiatry, neuropsychology, nursing, occupational therapy, physiotherapy, social work and speech therapy) whose role was to advise on the validity of culturally and linguistically modified items. The aim of the Culture Group (representatives from 22 cultural and linguistic groups) was to advise on the cultural and linguistic equivalence of proposed cognitive items, and to suggest modifications where relevant and appropriate. The iterative process used to conduct the two groups, and the aims and outcomes of each are described in Table 1.

ITEM-INCLUSION CRITERIA AND SCORING

The advisory groups endorsed 60 potential items for clinical testing. These items were reviewed by a cross-section of professional language interpreters, and finally

Table 2. Pilot items and cognitive domains

DOMAIN	ITEM - BRIEF DESCRIPTION
Orientation, memory and learning	Full name, country of birth, country now, city or town, month, day of week, 4-item animal recall, 4-item grocery recall, boy-big-dog sentence recall, circle pointing, children-fire story recall, design recognition and recall
Visuo-spatial and gnosis (including construction and drawing)	Body orientation, body placement, tell the time, pattern discrimination, copy (cube, lemniscates, intersecting pentagons, draw clock
Language and judgement	Animal generation, food generation, naming objects, 3-stage command, naturalistic conversation on childhood games, crossing the road, bathroom fall, kitchen fire, locked-out
Attention and perseveration/initiation	Symbol cancellation, days of week reversed, counting backwards from 20, finger tapping, ramparts
Planning and praxis	Simple maze, simple commands, fist-palm alternation task, fist-palm-side task
Insight, behavior and function	Self-rated insight, assessor-rated insight, behavioral rating by assessor, functional rating by assessor

by the study authors. The item-inclusion criteria were:

1. Good construct validity.
2. Independent of factors rich in cultural and linguistic variance or specific to a small number of cultures and languages.
3. Endorsed by interpreters.
4. Relatively short and easy to administer, without the need for complex stimuli or cue cards.

The 42 items which met the inclusion criteria were operationalized and retained for clinical testing in an outpatient setting (Table 2).

Stage 2 - Clinical testing of items

CLINIC PROCEDURE

The 42 items were tested on consecutive new referrals to the Geriatric Medicine outpatient clinic at Liverpool Hospital, in Sydney, Australia (January to December 2000). At the beginning of the clinic appointment, a research psychologist (JES) administered the items, blinded to patient diagnosis and clinical history. The items were randomly ordered to control for order effects, and professional health interpreters were used for all patients who did not speak English. Each patient was then comprehensively assessed by one of three geriatricians (JTR, DB and DAC), who ordered relevant investigations (including cerebral CT scans and laboratory tests), and implemented a standardized assessment of cognitive domains that excluded the 42 items for testing, so as to avoid

contamination. The administered the N Instrumental Active the Geriatric Depre Dementia Rating (Other Amnesic D Psychiatric Associa impaired but not decline), or demer review committee patients (or their p

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DATA ANALYSIS

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Stage 3 - Valid PROCEDURE

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contamination. The geriatricians also collected demographic information, and administered the Modified Barthel Index (MBI) (Wade and Collin, 1988), the Instrumental Activities of Daily Living (IADL) Scale (Lawton and Brody, 1969), the Geriatric Depression Scale (GDS) (Yeasavage *et al.*, 1983), and the Clinical Dementia Rating (CDR) Scale (Berg, 1988). DSM-IV criteria for Dementia and Other Amnesic Disorders were used to assign cognitive diagnoses (American Psychiatric Association, 1994). Each patient was classified as normal, cognitively impaired but not demented (including those with age-associated cognitive decline), or demented. The study protocol was approved by the institutional review committee of the South Western Sydney Area Health Service, and all patients (or their proxies) gave informed consent.

SAMPLE SIZE

The planned sample size of 150 for clinical testing was calculated on the basis of several factors. High sensitivity is more important than high specificity for a scale primarily intended for targeted screening. An estimated sensitivity of 80% (95% confidence interval (CI) of $\pm 10\%$) in a non-English speaking background (NESB) population needs a minimum of 60 NESB patients. Forty per cent of the patients were predicted to be from NESB countries (based on demographic data from our outpatient clinic). A minimum of 35 patients with normal cognition (not including those with age-associated cognitive decline) were required. The sample has to be sufficiently large to evaluate the independent contribution of approximately 15 items in a logistic regression model, with 8–12 subjects recommended for each item (Hosmer and Lemeshow, 2000).

DATA ANALYSIS

We measured the strength of the association between each of the 42 items and the cognitive diagnosis using the Spearman's rank-order correlation coefficient. Items with correlation coefficients less than 0.35 were excluded from further analysis. Where several of the remaining items measured the same domain, the item with the strongest correlation was retained, provided that it had the best construct validity, cultural appropriateness and clinical utility as judged by unanimous agreement of the authors. Multivariate logistic regression modeling was used to select the set of items that best predicted dementia, after adjustment for age.

Stage 3 – Validation of the RUDAS in a community setting

PROCEDURE

The predictive accuracy of the RUDAS was validated in 90 elderly, community-dwelling persons, selected at random from a large database of all patients referred to a large, multidisciplinary, community-based aged-care team between

1997 and 1999. Potential subjects were stratified into six groups according to language background (English-speaking background, Asian non-English speaking background, and non-Asian non-English speaking background) and cognitive diagnosis (demented and non-demented). Subjects from each cognitive group were then matched for age and sex, and invited by telephone to participate in the validation study. Invitations to participate continued until 15 participants were recruited to each of the six groups.

The research psychologist administered the RUDAS at the participant's home. Within several days, a geriatrician (JTR), blinded to the results of the RUDAS scale, assessed each subject at home or in the outpatient department. Both clinicians were blinded to previous cognitive diagnosis. To evaluate inter-rater reliability, each subject was assessed at home by both the research psychologist and a trained member of the aged care team, by coin toss. Approximately one week later, the psychologist administered the RUDAS again to measure test-retest reliability. Professional language interpreters were used where necessary (over the telephone and face-to-face) and written consent was obtained from all participants or their proxies.

SAMPLE SIZE

The sample size for Stage 3 was based primarily on showing good inter-rater and test-retest reliability in randomly selected community-dwelling persons. At least 65 subjects were required for an estimated correlation coefficient of 0.8 (with 95% CI $\pm 15\%$). We recruited at least 90 subjects to allow preliminary evaluation of the RUDAS in each of the language background subgroups.

DATA ANALYSIS

Logistic regression analysis was used to assess the independent contribution of the RUDAS components in predicting dementia. Frequency response analysis and receiver-operating characteristic (ROC) curve analysis were used to determine the extent to which item responses segregated normal and demented patients. As the items used different measurement scales, standardized regression coefficients were calculated to compare the strength of the relationships between the items and the cognitive diagnosis. The standardized coefficients were used to assign scores to each of the items, which were then added together to yield a total score for the RUDAS. The overall accuracy of the RUDAS and the optimal cut-point for the diagnosis of dementia were assessed using ROC curve analysis (Hanley and McNeil, 1982). Multivariate logistic regression modeling was used to assess the effect of age, gender, education, preferred language and other factors which may differentially affect performance, on the relation between the RUDAS scores and cognitive diagnoses. Inter-rater and test-retest reliability of the RUDAS was measured using the intraclass correlation coefficient (ICC) (Shrout

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Results

Stage 2 -

PATIENT

The 42 new referral patients with dementia (median age 75 years) in English-speaking background (NESB) co

Table 4. Item-diagnosis correlation coefficients

DOMAIN	ITEM	SPEARMAN'S COEFFICIENT
Memory	4-item grocery recall	0.501
Language	Animal generation	0.487
Memory	4-item animal recall	0.472
Memory	Children-fire story recall	0.461
Language	Food generation	0.422
Insight	Assessor-rated insight	0.418
Praxis	Fist-palm alternation task	0.417
Visuo-spatial (drawing)	Draw clock (Shulman)*	-0.393
Judgement	Crossing the road	0.388
Language		0.387
	Naturalistic conversation on childhood games	
Visuo-spatial (drawing)	Cube copying	0.379
Planning	Simple maze	0.377
Gnosis	Body orientation	0.374
Visuo-spatial (drawing)	Copy lemniscates	0.360
Judgement	Locked-out	0.354
Memory	Boy-big-dog sentence recall	0.354
Visuo-spatial (drawing)	Draw clock (Mendez)†	0.351

* Shulman clock scoring method {Shulman, 1986 #376; Shulman, 1993 #36}.

† Mendez clock scoring method {Mendez, 1992 #41}.

ITEM-DIAGNOSIS CORRELATIONS

Table 4 shows the Spearman's correlation coefficients between each of the items tested and the cognitive diagnosis. Items with correlation coefficients less than 0.35 were excluded from further analysis, and are not shown in Table 4. For simplicity, only the primary cognitive domain is shown for each item, although many items clearly measure more than one domain.

LOGISTIC REGRESSION

Table 5 shows the multivariate logistic regression model that best predicted dementia, after adjustment for age. Items significantly associated with a diagnosis of dementia included "4-item grocery recall", "crossing the road", "cube copying" and animal generation". Although "fist-palm alternation task" ($p = 0.065$) and "body orientation" ($p = 0.094$) failed to reach statistical significance, both were retained, as no other item (with a Spearman's coefficient of at least 0.35) measured praxis or gnosis. Age ($p = 0.17$) was not a significant predictor of dementia (after adjustment).

RUDAS ITEMS

The research psychologist administered the six RUDAS items. All items were administered sitting opposite the patient to control for level of difficulty. The

Table 5. Logistic r

VARIABLE
4-item grocery recall
Animal generation
Fist-palm alternation
Fist-palm (1)*
Fist-palm (2)*
Crossing the road
Cube copying
Body orientation
Age

* These refer to dumm

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Table 5. Logistic regression model: likelihood of dementia ($N = 152$)

SPEARMAN'S COEFFICIENT	VARIABLE	PARAMETER ESTIMATE	STANDARD ERROR	P VALUE	ODDS RATIO (95% CI)
0.501	4-item grocery recall	-0.97	0.31	0.002	0.38 (0.21-0.69)
0.487	Animal generation	-0.12	0.063	0.051	0.88 (0.78-1.00)
0.472	Fist-palm alternation task			0.065	
0.461	Fist-palm (1)*	3.55	1.53	0.020	34.83 (1.75-694.66)
0.422	Fist-palm (2)*	0.38	0.65	0.56	1.46 (0.41-5.18)
0.418	Crossing the road	-0.88	0.36	0.016	0.42 (0.20-0.85)
0.417	Cube copying	-0.50	0.24	0.039	0.61 (0.38-0.98)
	Body orientation	-0.58	0.35	0.094	0.56 (0.29-1.10)
-0.393	Age	-0.059	0.043	0.17	0.94 (0.87-1.03)
0.388					
0.387					

* These refer to dummy variables for the fist-palm alternation task.

order of items in the RUDAS validation instrument was determined on the basis of item characteristics and minimization of those factors which would be likely to increase test anxiety and make the questions confrontational and threatening for respondents. The final order of items was Memory (4-item grocery list), Gnosis (Body orientation), Praxis (Fist/palm alternating task), Visuo-spatial drawing (Cube copying), Judgement (Crossing the road), Memory Recall (Grocery list recall), and Language (Animal generation).

4-item grocery recall: The patient was required to remember four grocery items (tea, cooking oil, eggs and soap), after a maximum of five learning trials (to ensure item registration). If the patient was unable to recall any of the groceries, "tea" was used as a prompt.

Body orientation: The patient was asked to respond to the following commands (in the order stated):

1. "Show me your right foot".
2. "Show me your left hand".
3. "With your right hand, touch your left shoulder".
4. "With your left hand, touch your right ear".
5. "Point to or indicate my left knee".
6. "Point to or indicate my right elbow".
7. "With your right hand, point to or indicate my left eye".
8. "With your left hand, point to or indicate my left foot".

Fist-palm alternation task: The patient was asked to imitate a motor task, beginning by placing both hands palm down on the table (or the lap). One hand was then placed in a fist (in the vertical position) while the other remained palm down. Both hands were then simultaneously alternated between the two positions. Having learned the task, the patient was asked to maintain it at a moderate (walking) pace for approximately 10 seconds.

Table 6. Characteristics of 90 validation study patients by cognitive diagnosis

CHARACTERISTIC	NORMAL (n = 45)	DEMENTED (n = 45)	p VALUE
Age in years (mean \pm SD)	78.1 \pm 8.4	81.4 \pm 8.1	.082
Female (%)	82.2	73.3	.310
Years in Australia (median, Q1-Q3)*	42.0, 19.5-53.5	30.0, 18.5-69.0	.728
Preferred language other than English (%)	51.1	66.7	.134
Interpreter used (%)	44.4	66.7	.034
More than 6 years of education (%)†	51.1	35.0	.135
MBI score (median, Q1-Q3)	20.0, 18.0-20.0	11.0, 4.0-16.0	.0001
Lawton IADL score (median, Q1-Q3)	6.0, 3.5-8.0	0.0, 0.0-1.0	.0001
Factor potentially affecting performance (%)‡	42.2	52.3	.342
CDR			.0001
No dementia (%)	91.1		
Questionable dementia (%)	8.9	8.9	
Mild dementia (%)		20.0	
Moderate dementia (%)		31.1	
Severe dementia (%)		40.0	

Note. SD = standard deviation; Q1-Q3 = interquartile range; MBI = Modified Barthel Index; Lawton IADL = Lawton Instrumental Activities of Daily Living Scale; GDS = 15-point Geriatric Depression Scale; CDR = Clinical Dementia Rating scale.

* 30 (33.3%) patients were born in ESB countries; 20 (22.2%) were born in one of 11 European NESB countries; 30 (33.3%) were born in one of 12 Asian countries; 2 (2.2%) patients were born in Central or South America, 3 (3.3%) in Africa, and 5 (5.5%) in the Middle East.

† Education unknown for 5 subjects.

‡ All subjects were assessed for vision and hearing impairment, psychiatric disease (including depression), dysarthria and dysphasia, and musculoskeletal, and neurological diseases than might affect performance on the RUDAS.

Cube copying: This is a drawing task that requires the patient to copy a large line drawing of a cube.

Crossing the road: The patient is asked to describe how he or she would go about safely crossing a very busy street or similar thoroughfare where there is no pedestrian crossing or traffic lights. If the patient did not address two necessary components (looking for traffic and safety), a prompt – “is there anything else you would do?” was used.

Animal generation: The patient is asked to name as many new animals as possible in one minute.

Stage 3 – Validation of the RUDAS in a community setting

PATIENT CHARACTERISTICS

The 6-item RUDAS scale was validated in 90 elderly, community-dwelling persons. More than half of subjects had six or less years of education. Table 6 shows the characteristics of the participants by cognitive diagnosis (demented or non-demented). Those with dementia were similar to normal patients,

except for MBI sectional measures for participants with a preter.

NON-WEIGHTED 4-item grocery recall points). If a prominent grocery items was

Body orientation able to complete dementia perform commands should maximum score of

Cube copying: It assigned to the parallel internal lines on basis of multivariate copy” were not significant was simplified, all characteristics.

Crossing the road and safety) is score a maximum of one

Animal generation animals separated therefore decided concluded without score of eight pairs

WEIGHTED ITEA The standardized of the relationship scores, are shown (most strongly associated accordingly, for a

RELIABILITY OF Both the inter-rater the RUDAS were

by cognitive diagnosis

DEMENTED (n = 45)	p VALUE
81.4 ± 8.1	.082
73.3	.310
30.0, 18.5–69.0	.728
66.7	.134
66.7	.034
35.0	.135
11.0, 4.0–16.0	.0001
0.0, 0.0–1.0	.0001
52.3	.342
	.0001
8.9	
20.0	
31.1	
40.0	

Modified Barthel Index; Lawton
= 15-point Geriatric Depression

born in one of 11 European NESB
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/ cognitive diagnosis (de-
imilar to normal patients,

except for MBI score ($p = .0001$) and Lawton IADL score ($p = .0001$), functional measures known to correlate strongly with dementia. By default, NESB participants with dementia were assessed using a professional language interpreter.

NON-WEIGHTED ITEM SCORING

4-item grocery recall: Each correct response scored one point (maximum of four points). If a prompt was used, the maximum score was three. Registration of grocery items was not scored.

Body orientation: Response frequencies showed that normal participants were able to complete at least five instructions without difficulty. No subject with dementia performed more than four correctly, therefore it was decided that commands should continue only until five were completed correctly (for a maximum score of five).

Cube copying: Initially, one point for each of five “cube characteristics” was assigned to the patient’s drawing (3-dimensional drawing, based on a square, all internal lines drawn, all external lines drawn, perfect copy). However, on the basis of multivariate logistic regression, “3-dimensional drawing” and “perfect copy” were not significant predictors of cognitive diagnosis. The scoring method was simplified, allocating one point for each of the remaining three characteristics.

Crossing the road: Each of the two necessary components (looking for traffic and safety) is scored out of two points. Any component which is prompted scores a maximum of one point.

Animal generation: Based on ROC curve analysis, the ability to name eight animals separated most normal patients from those with dementia. It was therefore decided that once eight animals were named, this item could be concluded without testing for the entire one minute. There was a maximum score of eight points for the number of new animals named in one minute.

WEIGHTED ITEM SCORING

The standardized logistic regression coefficients used to compare the strength of the relationships between the items and the cognitive diagnosis, and the item scores, are shown in Table 7. Items with the largest standardized coefficients (most strongly associated with the cognitive diagnosis) were weighted and scored accordingly, for a total RUDAS score of 30.

RELIABILITY OF THE RUDAS

Both the inter-rater ($ICC = 0.99$) and the test-retest ($ICC = 0.98$) reliabilities of the RUDAS were very high.

Table 7. Item weights and scores using standardized coefficients

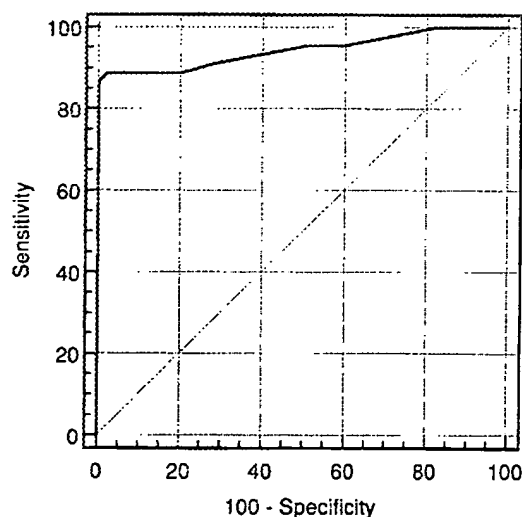
ITEM	STANDARDIZED COEFFICIENT (STANDARD ERROR)	ITEM SCORE
4-item grocery recall	1.8 (0.28)	8
Animal generation	1.6 (0.25)	8
Body orientation	1.3 (0.20)	5
Crossing the road	1.0 (0.16)	4
Fist-palm alternation task	0.5 (0.08)	2
Cube copying	0.2 (0.03)	3
Total		30

DIAGNOSTIC ACCURACY OF THE RUDAS

In our population, the diagnostic accuracy of the RUDAS (for detecting dementia based on DSM-IV criteria) was excellent, with an area under the ROC curve of 0.95 (95% CI 0.88–0.98) (Figure 1). Scores lower than 23 (the optimal cut-point based on the ROC curve) detected dementia with a sensitivity of 89% (95% CI 76%–96%) and a specificity of 98% (95% CI 88%–97%).

EFFECT OF GENDER, EDUCATION, PREFERRED LANGUAGE, AGE AND PERFORMANCE FACTORS

In the validation sample, gender ($p=0.18$), years of education ($p=0.20$), preferred language (English or otherwise) ($p=0.33$), and factors which may

**Figure 1.** ROC Curve for RUDAS

affect performance on
of dementia. Age ($p=$

Discussion

This study is the first heterogeneous sample work that has focused tests for use within or to develop instrumen populations modified originally developed in

The 6-item RUDA be administered by training (using videot translated into at lea structure or the forma were culturally diverse fair, thus avoiding the people from other bac

The RUDAS takes domains. In particular the road", "animal ge tioning (the ability to both directly and indi limitation of the M involving the frontal l non-verbal, written a subject's overall cogn municate a response is not over-emphasize

Gender, years of e differential performar multivariate logistic r instruments, where e instrument scores. A ($p=0.035$), but not age therefore remain standardized logistic relationships between associated with the c

coefficients

ICIENT

ITEM SCORE

8

8

5

4

2

3

30

the RUDAS (for detecting
dementia, with an area under the
ROC curve of 0.88. Scores lower than 23 (the
cutoff for dementia) had a sensitivity
of 95% (95% CI 88%–97%).

LANGUAGE, AGE AND

of education ($p = 0.20$),
, and factors which may

affect performance on a cognitive test ($p = 0.42$) were not independent predictors
of dementia. Age ($p = 0.04$) was a significant predictor in the same model.

Discussion

This study is the first to develop a multicultural cognitive test in a culturally
heterogeneous sample of elderly patients and addresses the gap in previous
work that has focused specifically on the development of cognitive screening
tests for use within or across discrete cultural groups. Most previous attempts
to develop instruments for use in culturally homogenous or heterogeneous
populations modified or translated scales (or their components) that were
originally developed in dissimilar groups.

The 6-item RUDAS is portable and requires no special equipment. It can
be administered by health care workers after approximately 40 minutes of
training (using videotape). Based on our experience, the RUDAS can be directly
translated into at least 30 other languages, without the need to change the
structure or the format of any item. Our study populations and advisory groups
were culturally diverse, allowing us to develop items we believe to be culturally
fair, thus avoiding the common approach of superimposing "western" ideas on
people from other backgrounds.

The RUDAS takes about 10 minutes to complete and tests multiple cognitive
domains. In particular, items relevant to frontal lobe function, such as "crossing
the road", "animal generation", and "cube copying", evaluate executive func-
tioning (the ability to initiate, plan and execute tasks relevant to daily living),
both directly and indirectly. Lack of evaluation of executive function is a major
limitation of the MMSE, which usually fails to detect meaningful deficits
involving the frontal lobes. The diverse response formats of the RUDAS (verbal,
non-verbal, written and praxic) allow more comprehensive assessment of a
subject's overall cognitive ability. Impairment in a domain necessary to com-
municate a response (but not necessarily causing important cognitive deficits)
is not over-emphasized, thereby reducing misclassification of cognitive capacity.

Gender, years of education, preferred language (English or otherwise), and
differential performance factors were not significant predictors of dementia in the
multivariate logistic model. This represents a major advantage over several earlier
instruments, where education, in particular, exerted a substantial influence on
instrument scores. Age was a significant predictor in our validation sample
($p = 0.035$), but not in the development sample ($p = 0.170$). The effect of
age therefore remains uncertain and needs further evaluation. We computed
standardized logistic regression coefficients to compare the strength of the
relationships between the items and the cognitive diagnosis. Items most strongly
associated with the cognitive diagnosis were weighted and scored accordingly.

In our validation sample, the RUDAS had excellent reliability (both inter-rater and test-retest) and diagnostic accuracy for detecting dementia based on DSM-IV criteria. Importantly, the RUDAS does not depend on informant history. Demented persons without informants are less likely to have carers, and are therefore at higher risk of the adverse consequences of dementia. The RUDAS items can be directly translated and are relevant to most cultures, strengths that should facilitate the use of the instrument in cross-cultural research.

Our study has several limitations. First we cannot exclude the possibility that we misclassified participants as demented or non-demented. Accurate diagnosis can be difficult, particularly in culturally heterogeneous populations (Erkinjuntti *et al.*, 1997; Prince, 2000). While there is no gold standard for dementia diagnosis, decisions based on DSM-IV criteria have the advantage of broad acceptance and good reproducibility (Baldereschi *et al.*, 1994; O'Connor *et al.*, 1996). Furthermore, each participant was comprehensively assessed by one of three geriatricians, who used a variety of information sources to make a diagnosis. The development sample was not random, and used patients attending a geriatric outpatient clinic. The possibility of bias within the sample is therefore far stronger than desirable. However, our validation sample had an even distribution of participants with normal cognition and those with dementia (50% in each group). Re-validation of the RUDAS in further community-based populations is desirable.

In summary, the 6-item RUDAS was developed and validated in culturally diverse samples. It is portable and easy to administer, with excellent reliability and diagnostic accuracy. It appears not to be affected by gender, years of education, differential performance factors and preferred language. It tests multiple cognitive domains, including those related to function of the frontal lobes. The RUDAS can be simply translated into other languages, without the need to change the structure or the format of any item. While designed primarily to detect dementia in the primary care setting, further work is needed in other settings, and in longitudinal studies to determine its sensitivity to change in cognitive function over time.

Acknowledgement

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Research report

Metalloproteinase ADAMTS-1 but not ADAMTS-5 is manifold overexpressed in neurodegenerative disorders as Down syndrome, Alzheimer's and Pick's disease

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Abstract

ADAMTS-1 is a disintegrin and metalloproteinase with thrombospondin 1 (TSP1)-like motifs with ubiquitous though variable expression. Natural substrates of this protease are proteoglycans as aggrecan and versican and null mutant mice propose a role for growth, fertility, organ structure and function. As the gene for this protein is encoded on chromosome 21 and maybe overexpressed due to the gene dosage hypothesis based upon the presence of a third chromosome in trisomy 21, we decided to study expression in Down syndrome (DS) brain and used brains of patients with Alzheimer's (AD) and Pick's disease (PD) as controls.

Frontal cortex of controls, DS, AD and PD were homogenized and extracted proteins were used for immunoblotting using antibodies against ADAMTS-1 and ADAMTS-5. ADAMTS-1-immunoreactivity was manifold increased in brain with DS and neurodegeneration, whereas ADAMTS-5 levels were comparable.

Overexpression of this metalloproteinase maybe specifically involved in proteoglycan degradation and handling in brain of patients with neurodegenerative disease which in turn may lead to or reflect pathological lesions in DS, AD and PD brain. The manifold overexpression of ADAMTS-1 may be used as marker protein for neurodegeneration.

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Theme: Disorders of the nervous system

Topic: Degenerative disease: other

Keywords: Metalloproteinase; ADAMTS; Neurodegeneration; Down syndrome; Alzheimer's disease; Pick's disease

1. Introduction

ADAMTS-1 is a disintegrin and metalloproteinase with thrombospondin 1 (TSP1)-like motifs which was originally reported as a gene highly expressed in the murine colon 26 cachecigenic tumor [11]. The human ortholog was named METH-1 and identified in cDNA screening using the TSP1 anti-angiogenic motif as bait [24].

METH-1/ADAMTS-1 consists of a signal peptide in the NH₂-terminal end, indicating a secreted protein (Swissprot

access number Q9UHI8), containing zinc metalloproteinase and disintegrin/cysteine-rich domains. The spacing region and the three TSP type 1 motifs found in the carboxy-terminal end of the protein are responsible for anchoring to the extracellular matrix [25]. The human pro-METH-1 protein is processed in two consecutive steps: first, furin deletion of the N-terminal prodomain resulting into an 87 kDa form. In the second step, specific metalloproteinases remove two thrombospondin repeats from the C-terminal end producing a 67 kDa form altering the affinity of the heparin protein and thus conferring different extracellular distribution and anti-angiogenic properties to each form [14,16].

Proteinase activity of METH-1/ADAMTS-1 was confirmed by the capability to form complexes with the high-

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affinity proteinase inhibitor $\alpha 2$ -M and by differential inhibition performed by the metalloproteinases inhibitors TIMP2 and TIMP3 [25,17]. At least two members of the chondroitin sulfate proteoglycan family bind to hyaluronan, the cartilage aggrecan and the wide distributed versican, have been reported so far as a substrates for this metalloproteinase [12,18]. ADAMTS-1 null mutant mice showed the importance of this enzyme for normal growth, fertility, organ structure and function [20].

Expression of ADAMTS-1 was predominantly detected by RNase protection assays (RPA) in the epithelium of the developing lung, pancreas, kidney and a subset of neurons in a temporally restricted manner; expression was highly reduced in adult organs [22]. The expression pattern of METH-1 was examined in human adult and fetal tissues by Northern analysis and a signal was detected in all tissues analysed, with a weak signal in both fetal and adult brain [24]. Interestingly, increased mRNA levels of ADAMTS-1 were detected in response to injured motor neurons and in kainate-sensitive brain regions after kainate administration in rats [19,26].

ADAMTS-5 (aggrecanase-2) is another disintegrin and metalloproteinase with thrombospondin 1 (TSP1)-like motifs also involved in the aggrecan and versican cleavage [1,23]. In adult human tissues examined with human cDNA probes, mRNA levels were detected in a variety of tissues, with a low signal in brain [9]. METH-1 and ADAMTS-5 proteins are the product of genes encoded on chromosome 21. We therefore analysed expression of METH-1 and ADAMTS-5 in frontal cortex of adult brains from Down syndrome, Alzheimer's disease, Pick's disease and controls by immunoblotting and detected more than fivefold overexpression of METH-1 in the neurodegenerated brains, whereas expression of ADAMTS-5 was comparable.

2. Materials and methods

2.1. Brain samples

Frontal cortex from patients with Alzheimer's disease ($n=6$; six males; 59.3 ± 6.4 years old), Down syndrome ($n=6$; six males; 57.8 ± 8.2 years old), Pick's disease ($n=4$; four males; 64.25 ± 5.50) and controls ($n=6$; six males; 60.2 ± 9.3 years old) were used in this study. Postmortem brain samples were obtained from the MRC London Brain Bank for Neurodegenerative Diseases, Institute of Psychiatry, King's College, UK, Dr. N. Cairns [10]. All the samples were stored at -70°C and the freezing chain was never interrupted.

2.2. Antibodies and control immunogen peptide

ADAMTS-1 antibody (Santa Cruz Biotechnology, USA), control immunogen ADAMTS-1 peptide (Santa Cruz

Biotechnology), and ADAMTS-5 antibody (Serotec, UK) were purchased.

The antibody against ADAMTS-1 is an affinity purified goat polyclonal antibody raised against a peptide mapping near the amino terminus of ADAMTS-1 of mouse origin (Santa Cruz Biotechnology). The information on the structure of the immunogen used for the immunization was not obtainable from the supplier. There is high homology (82%) between the mouse and the human protein.

The antibody against ADAMTS-5 is an affinity purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to the C-terminal region of ADAMTS-5 (Serotec).

2.3. Western blotting

Frontal cortex tissues ground under liquid nitrogen were homogenized in lysis buffer 1% SDS containing protease inhibitor cocktail tablets (Roche, Grenzach-Wyhlen, Germany), incubated 10 min at 37°C , boiled 10 min at 95°C and centrifuged at $8000 \times g$ for 10 min. The BCA protein assay kit (Pierce, Rockford, IL, USA) was applied to determine the concentration of protein in the supernatant. Samples (10 μg) were mixed with the sample buffer (100 mM Tris-HCl, 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 15 min and loaded onto a 12.5% ExcelGel SDS homogenous gel (Amersham Pharmacia Biotech, Uppsala, Sweden). Electrophoresis was performed with Multiphor II Electrophoresis System (Amersham Pharmacia Biotech). Proteins separated on the gel were transferred onto PVDF membrane (Millipore, Billerica, MA, USA) and membranes were incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 and 5% nonfat dry milk). Membranes were incubated for 2 h at room temperature with diluted primary antibodies: 0.04 $\mu\text{g}/\text{ml}$ for ADAMTS-1 and 1 $\mu\text{g}/\text{ml}$ for ADAMTS-5. After three times washing for 10 min with blocking buffer, membranes were probed with secondary antibodies coupled to horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL, USA) for 1 h. Membranes were washed three times for 10 min and developed with the Western LightningTM chemiluminescence reagents (PerkinElmer Life Sciences, Boston, MA, USA) [3].

Specificity of the immunoreaction on immunoblotting was tested by blocking the antibody (0.04 $\mu\text{g}/\text{ml}$) with the immunogen (2 $\mu\text{g}/\text{ml}$) for 17 h at 4°C in 500 μl TBS.

2.4. Statistics

The density of immunoreactive bands was measured by RFLPscan version 2.1 software program (Scanalytics, Fairfax, VA, USA). Between group differences were calculated by nonparametric Mann-Whitney U -test using GraphPad

Fig. 1. Wes were loaded with prima chemilum

Instat2 pr at $P < 0.05$

3. Results

We ana nases end ADAMTS

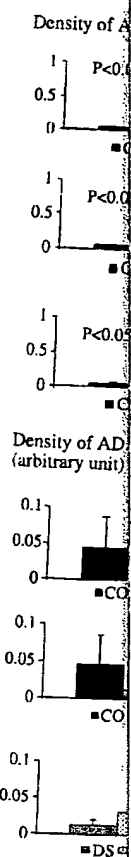


Fig. 2. Expression density of detected ADAMTS-1 and ADAMTS-5 in frontal cortex of adult brains from Down syndrome, Alzheimer's disease, Pick's disease and controls. Mann-Whitney U $P < 0.05$.

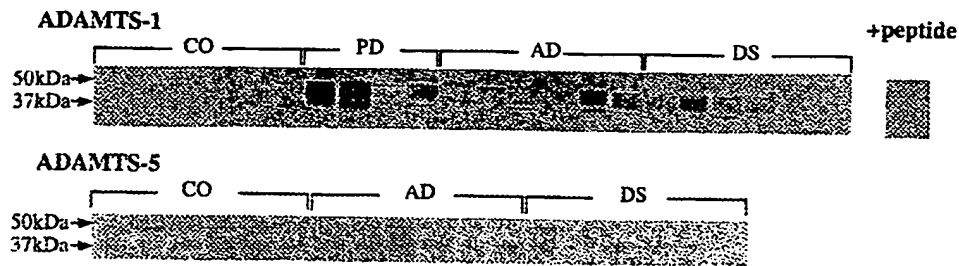


Fig. 1. Western blot analysis for ADAMTS-1 and ADAMTS-5 in cerebral cortex from adult brain with DS, AD, PD and controls. Denatured proteins (10 μ g) were loaded, separated on a homogeneous gel and transferred onto PVDF membrane. As described in Materials and methods, the membranes were incubated with primary and secondary antibodies, and immunoreactive bands (ADAMTS-1, 5037 kDa, ADAMTS-5, 50 and 37 kDa) were detected using chemiluminescence reagents. Blocking antibody by preincubation (+peptide) led to significantly lower density of immunoreactive bands.

Instat2 program and the level of significance was considered at $P < 0.05$.

3. Results

We analysed the expression levels of two metalloproteinases encoded on chromosome 21, ADAMTS-1 and ADAMTS-5, in brains of Pick's disease (PD), Alzheimer's

disease (AD), Down syndrome (DS) and control persons (CO).

A complex of several immunoreactive bands between 50 and 37 kDa was obtained with ADAMTS-1 antibody. The bands may represent protein polymorphisms/splicing variants or posttranslational processing. Total immunoreactive density of bands was tenfold increased in PD ($P = 0.0048$), around sevenfold increased in AD ($P = 0.013$) and fivefold increased in DS ($P = 0.0206$), whereas no significant differences were obtained between PD, AD and DS groups (Figs. 1 and 2).

Anti-ADAMTS-5 antibody detected two bands at 50 and 37 kDa whose density was statistically comparable between groups (Figs. 1 and 2).

The several bands may represent individual splicing variants or posttranslational modifications including oxidation, glycosylation, phosphorylation, methylation, nitrosylation, etc.

4. Discussion

Originally, we intended to test the gene dosage hypothesis predicting that ADAMTS whose genes are encoded on chromosome 21 were overexpressed in DS brain. In fetal DS both gene products were examined and while ADAMTS-1 was undetectable, although in the mouse it is expressed in developmental stage E11 and disappearing after E12 [21], ADAMTS-5 levels were comparable to controls [6]. We therefore determined ADAMTS-1 and ADAMTS-5 levels in adult brain and observed weak ADAMTS-1 immunoreactive bands in adult control brains. The major outcome was that ADAMTS-1 expression was manifold (about fivefold) overexpressed in DS frontal cortex. As adult DS present with AD neuropathological hallmarks from the fourth decade, observed data may not only reflect DS but also AD-related aberrant protein expression and we therefore had to test brains of patients with AD to rule out this effect. It turned out that also AD individuals showed a manifold approximately sevenfold overexpression as compared to controls. This finding made us examine another dementing neurodegenerative disorder, PD, to find out whether increased ADAMTS-1 levels in AD

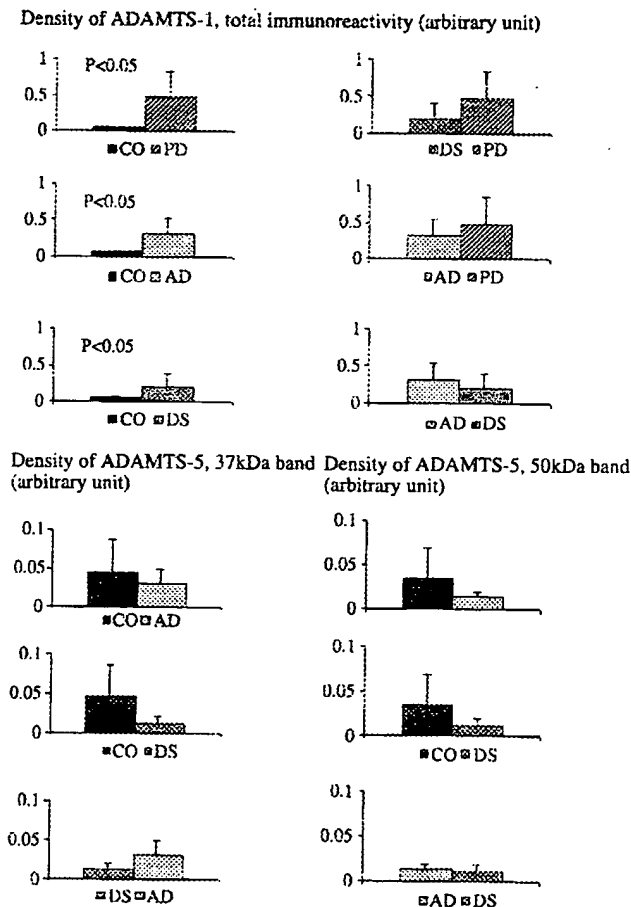


Fig. 2. Expression levels of ADAMTS-1 and ADAMTS-5 proteins. The density of detected bands was measured and calculated by nonparametric Mann-Whitney U -test, and the level of significance was considered at $P < 0.05$.

were representing specific findings for AD or simply reflected neurodegeneration-related changes. And indeed about tenfold overexpression of ADAMTS-1 was detected in PD frontal cortex.

Increased ADAMTS-1 in neurodegenerative disorders can be considered as specific though, as ADAMTS-5 levels were comparable between groups. Expression levels may not be confounded by differences in postmortem time or age as linear regression analysis did not reveal any significant correlations (data not shown). The specificity of the ADAMTS-1 antibody used was proven by the result of decreased intensity of bands obtained following peptide neutralisation control (data not shown).

Normalisation of ADAMTS levels versus housekeeping proteins as, e.g. cytoskeleton proteins or glyceraldehyde 3-phosphate dehydrogenase [13] were not carried out, as cytoskeleton protein derangement was observed in a nearly identical cohort of patients with neurodegeneration [15].

ADAMTS-1 and ADAMTS-5 were considered to exert identical functions, i.e. cleavage of several proteoglycans including aggrecan, versican and brevican and therefore the question arises why only ADAMTS-1 was increased in neurodegeneration. One explanation maybe substrate specificity in the brain, which is not known so far, although degradation of aggrecan has been described by both ADAMTS forms [17,23]. Unfortunately, no information on compartmentalisation or cell-specific distribution in the brain that may help with the interpretation of results is available. Localization in motor neurons and stimulation of ADAMTS-1 by interleukin (IL)-1 alpha, however, was reported and this points to a tentative mechanism [19]: Grimaldi et al. showed the strong association between IL-1 alpha and amyloid beta deposition in AD [8]. This would point to involvement of a neuroinflammatory reaction which was already described and is a widely accepted concept in neurodegeneration [7].

Abnormal accumulation of potential substrates (chondroitin sulfate proteoglycans) for ADAMTS-1 and ADAMTS-5 has been shown in senile plaques of AD: De Witt et al. [4] reported that chondroitin-4-sulfate was found both in senile plaques and neurofibrillary tangles in AD and chondroitin-6-sulfate in neurofibrillary tangles and the area around senile plaques. Díaz-Nido et al [5] reviewed and proposed a role of several glycosaminoglycans including chondroitin sulfate for favoring β -amyloid polymerisation in vitro and in vivo and indeed chondroitin and heparan sulfate proteoglycans were reported to localize at sites of β -amyloid deposition in brain of patients with AD [2,21] and the increase of proteoglycan degrading enzymes may be a mechanism to counteract excess β -amyloid polymerisation and deposition in AD and other neurodegenerative disorders [5].

Taken together, we have shown the manifold increase of metalloproteinase ADAMTS-1 but not ADAMTS-5, both encoded on chromosome 21, in brain of patients with neurodegeneration. This finding may represent or lead to

neurodegeneration identifying a marker protein and potential pharmaceutical target.

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REVIEW ARTICLE

Mild Cognitive Impairment

Focus on Diagnosis

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Abstract

Great interest is now devoted to elderly people with memory or other cognitive complaints who are not demented. The determination of this impairment from normality is difficult, because memory performance may decline slowly along the lifetime of the individual. On the other hand, the identification of dementia depends on the criteria used for dementia (DSM-IV or ICD-10). Furthermore, cognitive deterioration of the elderly appears to be heterogenous and may forerun not only Alzheimer's disease but also other forms of dementia. By applying a set of criteria for frontotemporal mild cognitive impairment, it was possible to identify, retrospectively, a series of patients with behavioral, affective, or speech symptoms suggestive of frontotemporal dysfunction and deficits in frontal lobe-dependent neuropsychological tests, but who have maintained activities of daily living and are not demented. These patients appear to have a high probability of progressing subsequently to dementia of the frontotemporal type. Several potential neuroprotective compounds are now being subjected to clinical trials. Should they be effective in delaying the progression to dementia, the need to detect and treat elderly people with cognitive impairment will become very important.

Index Entries: Mild cognitive impairment; Alzheimer's disease; aging; dementia; age-associated memory impairment; age-associated cognitive decline; frontotemporal dementia.

Introduction

As a consequence of the aging of the population, the number of patients with Alzheimer's disease (AD) and other dementias has been growing at an ever-increasing rate and will likely continue to increase in Western societies (Hebert et al., 2001). The concept of dementia implies the existence of multiple cognitive deficits sufficiently severe to cause impairment in occupational or social functioning, and represent a decline from a previously higher level of functioning (Diagnostic and Statistical

Manual [DSM-IV], American Psychiatric Association, 1994). However, many elderly people suffer from memory and other cognitive decline that is not severe enough to meet the criteria for dementia. Several different nosological concepts have attempted to describe this impairment in elderly people, for example, age-associated memory impairment (AAMI), mild cognitive impairment (MCI), and age-associated cognitive decline (AACD), just to mention a few (Ebly et al., 1995; Ritchie and Touchon, 2000; Blanchet et al., 2002). Independent of the particular concept, these elderly people have

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the quality of their lives significantly affected, do search for medical help and, very importantly, might be at high risk for further cognitive deterioration to the point of dementia (Petersen et al., 2001b). Any neuroprotective therapeutic intervention at this stage could have important consequences in the prevention of subsequent dementia (Petersen et al., 2001b).

Certainly, the cognitive deterioration of the elderly is bound by normality on one side and dementia on the other. Unfortunately, both boundaries have been difficult to establish.

The Difficulty in Defining Normal Memory

The boundary with normality is difficult to define, particularly because memory performance declines in older subjects (Small et al., 1999b; see also Balota et al., 2000). However, the notion that as people grow old (how old?), they suffer a precipitous impairment in memory might not be correct. In fact, memory complaints are also frequent in young adults (Ponds et al., 1997). Furthermore, a slow decline in memory performance can be demonstrated from young adulthood. We recently studied 100 healthy subjects, blood donors (18–63 yr old, 10.3 ± 5.3 yr of education), who performed several neuropsychological tests. These included the California Verbal Learning Test (CVLT; Delis et al., 1987), which is a very demanding test for declarative memory. There was a slow and steady decline in total number of correct responses in the CVLT with age. Most interestingly, this descent began as early as the third decade of life. In a linear regression model, memory decline was associated with aging but not with other possible variables, namely sex, education, depression, anxiety, and the score in a subjective memory complaints scale. These results emphasize the importance of longitudinal assessment of cognitive functions and, in particular, memory to better understand the decline that becomes apparent in the aged.

The Difficulty in Defining Dementia

We studied a consecutive series of 897 subjects (61.8 ± 10.9 yr old, 54.3% females, 5.3 ± 4.3 yr of education) referred to the Laboratory of Language, Faculty of Medicine of Lisbon, with possible cognitive impairment. These subjects were evaluated for the presence of dementia by applying two commonly used criteria: one from the DSM-IV (American

Psychiatric Association, 1994), and the other from the International Classification of Diseases (ICD-10, World Health Organization, 1992). Interestingly, 82.6% of the subjects were demented according to the DSM-IV criteria, and only 63.8% following the ICD-10 criteria (Guerreiro, 1998). Concordance between the two criteria was good, as they agreed in classifying 81.3% of the subjects. The discordance between the criteria was essentially attributable to subjects who were considered to be demented with the DSM-IV criteria but not with the ICD-10 criteria. The ICD-10 criteria are thus more stringent in diagnosing dementia, essentially because they require the presence of thinking impairment and the presence of symptoms for at least 6 mo (Guerreiro, 1998). That is, the diagnosis of dementia in a substantial number of patients is critically dependent on the criteria used to define dementia.

Cognitive Decline of the Elderly Might Correspond to Different Clinical Entities

Heterogeneity of the cognitive decline in the elderly was shown by cluster analysis techniques (Ylikoski et al., 1999). Certainly there are different clinical situations or, possibly, stages from normality all the way along to dementia. Thus, in community-based epidemiological studies, about 40% of people older than 50 have AAMI, that is, complaints of memory and an abnormal performance in memory tests when compared to young people, although they maintain adequate intellectual function and show no signs of dementia (Koivisto et al., 1995). A subgroup of these patients might correspond to the criteria of MCI, that is, they present with memory complaints (preferably corroborated by an informant), and the neuropsychological tests show abnormal memory for age and education but normal general cognitive function. Importantly, they maintain their activities of daily living and are not demented (Petersen et al., 1999). In contrast with AAMI, in elderly patients with MCI, the memory performance is compared with the norms for old, and not young, people. These aged people who meet MCI criteria appear to be at high risk to deteriorate cognitively. Thus, in a memory clinic setting, as many as 80% will actually develop dementia, usually AD, in 6 yr (Petersen et al., 2001a). Patients with MCI are also at higher risk for death (Bennett et al., 2002). The prognosis of MCI in a community setting, however, appears to be less consistent and less bleak (Ritchie et al., 2001).

In clinical practice, many elderly people have deficits not only in memory but also in other cognitive domains as compared with norms for elderly people, thus not fulfilling the criteria strictly for MCI in the sense of amnesic MCI. They might correspond to AACD (Levy, 1994), which stipulates a decline in any area of cognitive function in comparison with age-matched controls, or to the more recently proposed concept of MCI with multiple domains slightly impaired (Petersen et al., 2001a). In one study based in the community, elderly people with AACD, but not those with MCI and deficits restricted to memory, have a high risk of progression to dementia (Ritchie et al., 2001).

Obviously, the limits and meaning of these clinical entities are still not entirely clear. A substantial amount of data has been recently provided for patients with amnesic MCI. However, more must be known about the anatomical and pathological correlates and prognosis of memory complaints in elderly people who do not attain the criteria for MCI. For this purpose, elderly people could be stratified from a psychometric (and functional) point of view. Some elderly would have a performance in neuropsychological tests within (or above) the norms for young people and no cognitive complaints (they would correspond to optimal aging). Others would have a less satisfactory performance in neuropsychological tests but still within the norms for aged people, with or without mild cognitive complaints.

Cognitive Deterioration in the Elderly Might Predict a Number of Different Neurodegenerative Disorders

Alzheimer's disease (AD) is the most common form of dementia. It is thus expected that most cases of MCI that progress would eventually result in the diagnosis of AD. However, we would anticipate that other forms of neurodegenerative disease, usually expressed as a dementia syndrome, should also have a more or less insidious prodromal phase before dementia. Such would be the case of frontotemporal dementia and Lewy body dementia, among other neurodegenerative disorders (Petersen et al., 2001a).

We have recently proposed criteria for the diagnosis of mild cognitive/executive impairment (de Mendonça et al., 2002). These criteria are (1) the presence of behavioral, affective, or speech symptoms suggestive of frontotemporal dysfunction (adapted from the criteria for frontotemporal

dementia; The Lund and Manchester Groups, 1994; Neary et al., 1998)—typically, these patients exhibited apathy, disinhibition, irritability and aggressiveness, untidiness, difficulties in decision making, obsessions, and lack of concern for others; (2) a deficit in frontal lobe-dependent neuropsychological tests (memory tests might also be affected); (3) maintenance of the activities of daily living; (4) the absence of dementia; and (5) computed tomography or nuclear magnetic resonance (NMR) scan normal or showing frontotemporal atrophy. Other neurological or psychiatric disorders should be excluded by clinical history, brain imaging, and laboratory tests. By systematically searching the Dementia Clinics and Laboratory of Language databases, in the Faculty of Medicine of Lisbon, we identified 7 subjects retrospectively (61.0 ± 13.0 yr old, 5 females) who fulfilled these criteria. It was possible to follow these patients until the present. Five of the 7 patients developed dementia of the frontotemporal type within 1.6 ± 1.0 yr. The two patients who did not were followed for a shorter period, and continued observation will determine if these patients will in fact progress to frontotemporal dementia. Based on these preliminary findings, the sensitivity and specificity of the proposed diagnostic criteria should be determined in a large prospective series.

Exams to Assist in the Diagnosis of Cognitive Deterioration in the Elderly

Several ancillary exams have been proposed to aid in the diagnosis of cognitive deterioration and specifically to establish the prognosis of elderly people with cognitive complaints.

Neuropsychological examination is necessary to establish the profile and the severity of cognitive deficits. Different neuropsychological tests have been proposed to predict progression to dementia but were not in agreement in separate studies (Flicker et al., 1991; Tierney et al., 1996a). Rather expectedly, the severity of the memory deficit (Visser et al., 1999) and the presence of disturbed cognitive functions other than memory (Bozoki et al., 2001), possibly revealing a more advanced stage of cognitive impairment, predict progression to dementia. Also, not surprisingly, the presence of biochemical or genetic markers associated with AD, namely high levels of tau and low levels of amyloid β ($A\beta_{42}$) in cerebrospinal fluid and the presence of

the apolipoprotein E (ApoE) $\epsilon 4$ genotype, increase the probability of progression to dementia (Tierney et al., 1996b; Andreasen et al., 1999).

Great interest has been raised by the use of NMR to detect early changes in brain structures, which are important for memory functions and are known to be affected in AD. Volumetric study of the hippocampus in subjects with MCI detected hippocampal atrophy, which could, to some extent, predict progression to dementia (Jack et al., 1999). Interestingly, recent data show that hippocampal atrophy correlates with the neuropathologic stage in AD (Gosche et al., 2002) but is not specific for AD pathology. Atrophy of this brain structure can also be found in cognitively impaired patients with other neurodegenerative disorders (Jack et al., 2002). Several other advanced NMR techniques, including functional NMR (Small et al., 1999a), spectroscopy (Kantarci et al., 2000), diffusion-weighted NMR (Kantarci et al., 2001), and magnetization transfer (Kabani et al., 2002; van der Flier et al., 2002), have detected changes in the hippocampus and related structures in MCI. It is not yet clear whether the combination of these NMR techniques might be superior in detecting the early changes associated with cognitive impairment in the aged. Furthermore, the possibility of combining several demographic, genetic, neuropsychological, and radiological markers has been suggested (Visser et al., 2002), but again the clinical usefulness of this approach remains to be established.

The Importance of Diagnosing Cognitive Deterioration in the Elderly

It is now accepted that patients with MCI should be identified and monitored for cognitive and functional decline because of their increased risk for subsequent dementia (Petersen et al., 2001b).

Cognitive decline in elderly people can have different causes (Small, 2001). Importantly, a specific and treatable cause might be found, for instance, an endocrine or vitamin deficiency, cerebrovascular disease, sensory deficit, poor cognitive stimulation, medications, and depression (Knopman et al., 2001). In clinical practice, the identified abnormality may not be the main cause responsible for the cognitive symptoms as compared with the neurodegenerative pathology. Yet detection of the underlying cause is still important as it at least contributes to cognitive deterioration and is amenable to treatment.

Today, several compounds are being subjected to clinical trials to evaluate their efficacy in preventing

or slowing the progression of MCI to dementia (Scorer, 2001). Should they prove effective, the detection and treatment of mild cognitive decline in the elderly will probably become a public health priority in the near future. The very ancient and everlasting aim of prolonging youthfulness might now be closer.

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